Responses of Cultured Tobacco Cells to Cryptogein, a Proteinaceous Elicitor from Phytophthora cryptogea

Possible Plasmalemma Involvement

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ABSTRACT

In culture, the phytopathogenic fungus Phytophthora cryptogea secretes a protein which elicits hypersensitive-like necroses and protects tobacco plants against invasion by the pathogen Phytophthora parasitica var. nicotianae. This protein, named cryptogein, has been purified and its amino acid sequence determined. In this work, we studied the effect of cryptogein on tobacco cell suspension cultures. Cryptogein was lethal at about 0.10 micromolar. When added at sublethal doses, it elicited the production of ethylene and phytoalexins. It also induced a rapid increase in pH and conductivity of the extracellular medium without affecting the integrity of the plasma membrane. Cryptogein reduced the fusicoccin-induced acidification of the extracellular medium. The concentration which inhibited the fusicoccin response by 50% was 0.8 nanomolar, while 1 micromolar erythrosine B, an ATPase inhibitor, was needed to produce the same inhibition. However, cryptogein did not inhibit the activity of a purified plasma membrane ATPase. Results of binding studies with whole cells suggested the presence of elicitor-binding sites with a high affinity for cryptogein. The involvement of the plasma membrane during the initial interaction between elicitor and cells is discussed.

The interaction between plants and incompatible pathogens leads to the HR defined as a rapid and localized necrosis of a few cells at the site of attempted invasion resulting in protection of the plant against the pathogen. Presumably, these pathogens release elicitors that induce the HR. However, definitive proof that known elicitors are responsible for the HR is lacking.

The HR includes a number of phenomena. Net efflux of electrolytes is among early plant cell responses (13, 18). It may result from the activation of a plasma membrane K⁺ efflux/H⁺ influx exchange (4, 5). Membrane potentials are altered during the HR (23) or in response to elicitors (21, 24). In addition, a redox system which appears to be associated with plasmalemma generates superoxide anions (16, 17).

Thus, the plasma membrane seems to be involved in signal transmission leading to the induction of defense reactions (14, 28, 30). Among the long-term responses associated with the HR are accumulation of phytoalexins (15) and the induction of an enhanced rate of ethylene synthesis (11, 26). However, the biochemical mechanisms involved in the HR are not yet understood, and the trigger for hypersensitive cell death remains to be identified.

A major limitation in studying plant cell responses to microorganisms is the few defined and fully characterized elicitors available. In the incompatible interaction between Phytophthora cryptogea and tobacco, an original feature has been reported: when P. cryptogea is inoculated to the stem of its non-host tobacco plant, fungal colonization is restricted and a local necrosis occurs which is typical of an HR (8). However, necroses also develop on the leaves at a distance from the inoculation court (8). We believe that they are also hypersensitive-like reactions (despite their unusual location) because both the local and the distant necroses occur simultaneously (24–36 h post inoculation) and do not evolve afterward. From the culture filtrate of P. cryptogea, a protein, CRY (mol wt 10,323), has been isolated (6). When applied on tobacco stems, it causes similar necroses (25). It also elicits PR protein accumulation and protects tobacco plants against invasion following inoculation with the compatible pathogen Phytophthora parasitica var. nicotianae (8, 9). It has been purified and its amino acid sequence determined (25). Therefore, CRY appears to be a potential HR elicitor. Its interaction with tobacco cells is a convenient model to study the HR, and the use of a highly purified elicitor, rather than a crude extract, should facilitate further biochemical analysis.

Our major aim is to study the role of plasma membrane in the HR in the interaction of CRY with tobacco cells. However, preliminary studies on physiological responses of whole cells are needed: (a) to compare physiological responses of tobacco to cryptogein with those reported for HR and prove that cryptogein is indeed an elicitor of HR, and (b) to provide a physiological foundation for biochemical analyses of the tobacco response to CRY. In this work, we have examined the production of ethylene and of the phytoalexin capsidiol by CRY-treated cells and studied the changes in extracellular pH and conductivity. The possible involvement of the plasma membrane during the initial interaction between CRY and

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2 Abbreviations: HR, hypersensitive reaction; CRY, cryptogein; EB, erythrosine B; FC, fusicoccin.
cells is discussed based on a comparison of the effects of CRY with a known ATPase inhibitor and from experiments of cell binding determinations.

**MATERIALS AND METHODS**

**Materials**

Suspension cultures of tobacco cells were grown in the medium of Chandler et al. (10) on a rotary shaker. Cell growth was estimated by the increase in dry weight during a 96-h period.

CRY was purified according to the method of Ricci et al. (25) and added to suspension cultures as an aqueous solution.

FC was a generous gift of Pr. E. Marrè (Dipartimento di Biologia, Università degli studi di Milano, Italy).

**Ethylene and Capsidiol Production**

Three days after subculturing, 10-mL aliquots (1 g fresh weight) of the suspension were transferred to sterile 25-mL Erlenmeyer flasks, and the appropriate amounts of a sterile solution of the elicitor were added.

For ethylene measurements, the flasks were sealed with rubber caps. Samples of internal gas (1 mL) were withdrawn and analyzed by gas-liquid chromatography (Delsi DI 700 equipped with a flame ionization detector and a packed column Porapak Q 80-100 2 m × 3 mm; N2 carrier gas 30 mL/min; injector and detector at 120°C; column at 35°C). A calibration curve was established with ethylene standards (Aldrich).

For capsidiol determination, the extracellular medium was extracted with dichloromethane then analyzed by gas-liquid chromatography (packed column 3% OV-225 on chromosorb W100-120, 1.5 m × 3 mm; N2 carrier gas 30 mL/min; injector at 235°C; detector at 255°C; column at 180°C). Quantities were estimated from a standard curve of an authentic sample of capsidiol.

**Extracellular pH and Conductivity**

Cells from cultures in log-phase growth were collected by filtration and washed and resuspended (0.1 g fresh wt/mL) in 175 mM mannitol, 0.5 mM CaCl2, 0.5 mM K2SO4, and 2.0 mM Mes buffer, adjusted to pH 5.75 with NaOH (19). Aliquots of 20 mL were transferred to 50-mL Erlenmeyer flasks and preincubated for 2 h on a shaker (150 rpm) at 25°C.

![Figure 1. Time course of ethylene and capsidiol production by tobacco cells elicited by CRY. Each assay contained 0.1 g of cell fresh weight/mL. (A) Ethylene production (○) control, (●) 10 nM CRY, and (■) capsidiol production (■) control, (□) 12.5 nM CRY.](image)

![Figure 2. Rate of ethylene and capsidiol production by tobacco cells elicited by CRY. Each assay contained 0.1 g of cell fresh weight/mL. Ethylene (○) and capsidiol (■) production. Rates of ethylene and capsidiol production were calculated from time course experiments during 2 to 4 h and 6 to 18 h for ethylene and capsidiol, respectively.](image)

![Figure 3. Effect of CRY on tobacco cell growth. Cell growth was estimated by the increase in dry weight over a 96-h period. The doubling time of control cells was 48 h.](image)
Mes buffer was titrated and its buffering capacity was 20.6 μeq H⁺/pH unit, in the pH range 5.6 to 6.7 corresponding to the experimental conditions. Extracellular pH and conductivity were monitored for the first 30 min and initial velocities determined.

**Radioiodination of CRY**

CRY was labeled with ¹²⁵I as described by Salesse et al. (27) with iodogen (Pierce) as the catalyst and ¹²⁵I (Amersham). The reaction mixture containing [¹²⁵I]cryptogein was loaded onto a G-25 Sephadex column equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.5 g/L BSA. The [¹²⁵I]CRY eluting in the void volume was collected and stored at −20°C (specific activity was 150 μCi/nmol).

**Binding Assay**

Tobacco cells were removed from the medium by filtration and resuspended in a fresh culture medium (60 mg fresh weight/mL). After equilibration (2 h, 25°C), the suspension was divided into 2-mL fractions and CRY was added (50,000 dpm [¹²⁵I]CRY/assay). Cells were incubated on a shaker for 30 min at 4°C, withdrawn by filtration through a Whatman GF/A filter, washed with 5 mL of distilled water, vacuum desiccated (30 s), and weighted before counting in Beckman Ready Safe cocktail.

**RESULTS**

**Ethylene and Capsidiol Production**

When assayed on tobacco cell suspensions, CRY induced ethylene and capsidiol production. The time course of ethylene production in response to treatment with 10 nM CRY is shown in Figure 1A. Production started after a 120-min lag period, then increased linearly. Similar responses of capsidiol accumulation in the extracellular medium were obtained at 12.5 nM CRY (Fig. 1B). The lag period was longer (4–5 h), and the production increased over a 24-h period. Capsidiol accumulated mainly in the extracellular medium, since the amount extracted from cells represented 8.2% of that from the culture medium (incubation 18 h, 50 nM CRY). From time course experiments, we calculated the rate of ethylene production for 2 to 4 h and capsidiol production for 6 to 18 h. The concentration dependency of ethylene and capsidiol production is shown in Figure 2. The rate of ethylene production was maximal at 10 nM and then declined slightly at higher concentrations. Cells accumulated capsidiol when treated with CRY concentrations as low as 2.5 nM. This accumulation reached a plateau at 12.5 nM.

**CRY Toxicity**

Tobacco cells exposed to CRY turned brown after a few hours of exposure to concentrations higher than 50 nM. After 96 h incubation, cell growth was inhibited. This inhibition increased linearly with the elicitor concentration. Concentra-

**Table I. Inhibition of the Fusicoccin-Stimulated Cell Proton Extrusion by CRY**

At zero time FC, EB, and CRY were added to tobacco cells (2 g) resuspended as described in "Material and Methods."

<table>
<thead>
<tr>
<th>Addition of</th>
<th>H⁺</th>
<th>Percent of FC Response</th>
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<tbody>
<tr>
<td></td>
<td>neq/min</td>
<td></td>
</tr>
<tr>
<td>FC, 10 μM</td>
<td>50.9</td>
<td>100</td>
</tr>
<tr>
<td>− FC</td>
<td>4.28</td>
<td>8.4</td>
</tr>
<tr>
<td>+ EB, 1 μM</td>
<td>25.4</td>
<td>49.9</td>
</tr>
<tr>
<td>+ EB, 5 μM</td>
<td>12.7</td>
<td>24.9</td>
</tr>
<tr>
<td>+ CRY, 0.5 nM</td>
<td>42.7</td>
<td>83.8</td>
</tr>
<tr>
<td>+ CRY, 1 nM</td>
<td>16.2</td>
<td>31.8</td>
</tr>
<tr>
<td>+ CRY, 5 nM</td>
<td>12.2</td>
<td>23.9</td>
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tions above 100 nm were lethal (Fig. 3). Cell growth was inhibited by 50% (IC50 value) at 50 nm.

**Extracellular pH and Conductivity**

At concentrations below 50 nm, extracellular pH and conductivity increased within 2 to 3 min after addition of CRY (Fig. 4). These responses were observed at concentrations as low as 0.5 nm and were maximal at about 20 nm (Fig. 5). Under these conditions, CRY did not alter the cell membrane integrity, determined as described previously (7) from the amount of fluorescein released into the extracellular medium by cells preincubated with fluorescein diacetate (data not shown). In order to further study the modifications in extracellular pH, we treated cells with FC, which is known to stimulate cell proton extrusion (20), and with EB, which inhibits electrogenic H+ extrusion (12). When added at the same time as FC, EB, or CRY inhibited the proton extrusion induced by FC (Table I). Inhibition was greater at higher concentrations of EB and CRY. However, CRY appeared to be a more effective inhibitor than EB; about 75% inhibition was reached at 5 nM CRY, while 5 μM EB was required to achieve the same amount of inhibition (Table I). The concentrations which inhibited the FC response by 50% were 0.8 nm and 1 μM for CRY and EB, respectively.

In order to test the possibility that H+ ATPase is a primary target for CRY, we prepared tobacco plasma membrane purified by two-phase partitioning. Specific activity of the plasma membrane ATPase obtained was 4.4 μmol ATP hydrolyzed/min·mg protein. At 1 μM cryptogein, *i.e.* at a concentration 1250-fold higher than the IC50 value of the FC-induced proton extrusion by cells, 98% of the ATPase activity was recovered.

**Binding of [125I]CRY to Tobacco Cells**

The binding of [125I]CRY to tobacco cells decreased as progressively higher concentrations of unlabeled CRY were added until it reached the nonspecific binding level. Competitive displacement of the [125I]CRY from cells by unlabeled ligand showed an IC50 value of 500 nm (Fig. 6). A Scatchard plot of the specifically bound values shown in Figure 6 gave a straight line (Fig. 7), suggesting the presence of a single affinity class of binding sites. Figures 6 and 7 represent typical results, the apparent Kd value, as determined from three independent radioligand saturation experiments, was 214 ± 39 nm. The number of binding sites for the [125I]CRY was found to be 174 ± 2 pmol/g fresh weight.

**DISCUSSION**

Data reported here describe the responses of plant cells to CRY and confirm that this well characterized protein is an elicitor of HR. Its primary structure and its effect on tobacco plants (induction of hypersensitive-like necrosis and protection against the pathogen *Phytophthora parasitica var. nicotianae*) have been previously reported (25). Increases in extracellular pH and conductivity and production of ethylene and capsidiol were observed only when tobacco cell suspensions were incubated with cryptogein. Similar responses, regarded as characterizing the HR, have been reported when tobacco cells are treated for 1–2 h with *Pseudomonas syringae* (4, 19). However, in our system, substantial increases in pH and conductivity occur within the first few minutes of exposure of tobacco cells to cryptogein (Fig. 4). By contrast, we have not observed production of ethylene or capsidiol production within the first 2 h after addition of the elicitor. These responses are specific for tobacco cells, since sycamore, soybean, and tomato cells were insensitive to cryptogein (data not shown).

It has been reported that these early events (ion leakage and extracellular alkalization) are correlated with a decrease in intracellular pH (22) and a depolarization of the plasma membrane (23, 24). These responses could result from either
a general perturbation of membrane function or from an alteration of specific proteins. Ullrich-Eberius et al. (29) reported that change in pH results from HCO₃⁻ efflux, and that electrolyte loss is nonspecific (other ions in addition to K⁺) and results from general membrane damage by active oxygen and lipid peroxidation. In contrast, Atkinson et al. (3) suggested the involvement of a K⁺ channel, and Atkinson and Baker (2) showed that plasmalemma H⁺-ATPase activity is required for the K⁺/H⁺ exchange response but is not necessarily involved in HR initiation. We found that CRY did not affect the plasma membrane integrity, although it induced a very rapid increase in pH and conductivity of the extracellular medium and decreased the FC-induced acidification of the extracellular medium. These observations suggest that plasma membrane ATPase might be required for the early steps of the HR. However, H⁺ ATPase appears insensitive to CRY, and thus we hypothesize that the elicitor could indirectly inhibit the plasma membrane ATPase via a binding to elicitor-binding sites in the same way as FC (1). Specific binding characteristic of a receptor has been shown for glucan-type elicitors (14, 28, 30). Binding assays reported here are consistent with the presence of elicitor-binding sites for CRY. The observed binding was saturable and susceptible to displacement by unlabelled ligand. Further experiments are needed to locate the elicitor-binding sites of CRY and to determine whether plasmalemma ATPase could be a primary target for this elicitor. The use of a purified proteinaceous elicitor should facilitate investigations on elicitor-binding sites and on the physiological modifications involved in the early phase of the HR.

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LITERATURE CITED

Structure and activity of proteins from pathogenic fungi *P. topophthora* eliciting necrosis and acquired resistance in tobacco. Eur J Biochem 183: 555-563


