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Protein Phosphorylation Is Induced in Tobacco Cells by the Elicitor Cryptogein[1]

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Changes in plasmalemma ion fluxes were observed when tobacco (*Nicotiana tabacum*) cells were treated with cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*. A strong alkalization of the culture medium, accompanied by a leakage of potassium, was induced within a few minutes of treatment. These effects reached a maximum after 30 to 40 min and lasted for several hours. This treatment also resulted in a rapid, but transient, production of activated oxygen species. All these physiological responses were fully sensitive to staurosporine, a known protein kinase inhibitor. Furthermore, a study of protein phosphorylation showed that cryptogein induced a staurosporine-sensitive phosphorylation of several polypeptides. These data suggest that phosphorylated proteins may be essential for the transduction of elicitor signals.

The HR is a characteristic response of plants to incompatible pathogens both in race-cultivar and in nonhost interactions. It is defined as the necrotization of a more or less extended group of plant cells around the site of pathogen ingress associated with a limitation of parasite invasion. The HR is accompanied by important changes in plant cell metabolic activities, including the activation of defense mechanisms such as phytoalexin synthesis, and may also lead to acquired resistance to further infection.

Early responses of cells undergoing an HR include a number of phenomena such as a net efflux of electrolytes (Goodman, 1968), which may result from the activation of a plasma membrane K^+/H^+ exchange (Atkinson et al., 1986; Atkinson and Baker, 1987; Baker et al., 1987), an alteration of membrane potential (Pavlovkin and Novacky, 1986; Pellisier et al., 1986; Mayer and Ziegler, 1988), and the activation of a plasma membrane redox system that generates activated oxygen species (Doke, 1983; Doke and Ohashi, 1988). Various molecules, called elicitors, that induce defense reactions in plants have been shown to trigger several of these responses, and some elicitors induce the HR (Ricci et al., 1993). The plasma membrane appears to be involved in the transmission of elicitor signals (Yoshikawa et al., 1983; Schmidt and Ebel, 1987; Cosio et al., 1988) and there is evidence for elicitor-binding proteins in the plasma membrane (Schmidt and Ebel, 1987; Blein et al., 1991; Cheong and Hahn, 1991; Frey et al., 1993), but the transduction pathways remain unknown. Recently, rapid changes in protein phosphorylation have been reported to be involved in the transduction process of some elicitor signals (Felix et al., 1991; Sacks et al., 1992). However, such observations have not yet been reported for elicitors of the HR.

For the past few years, we have been studying the biological effects of elicitins, which are proteinaceous elicitors from *Phytophthora* species, and particularly cryptogein, a polypeptide produced by *Phytophthora cryptogea*. This elicitor causes HR-like necrosis in tobacco (*Nicotiana tabacum*) plants at the site of application (stem or roots) and also on distant leaves (Ricci et al., 1989). When added to the culture medium of tobacco cells, cryptogein induces phytoalexin accumulation and ethylene production (Milat et al., 1990). Its primary target may be located on the plasmalemma because (a) it causes a very rapid increase in the pH and conductivity of the extracellular medium, (b) it suppresses fusicoccin-induced acidification of the extracellular medium, and (c) it binds to specific sites with a high affinity (Blein et al., 1991). However, cryptogein does not inhibit ATPase activity of a purified plasma membrane from tobacco (Blein et al., 1991). This latter finding suggests that the elicitor could indirectly inhibit the plasma membrane ATPase via binding to specific sites and consequently led us to examine the potential signal transduction pathway(s) associated with its effects. Therefore, we considered the possibility that a phosphorylated protein is involved in the responses of cryptogein-treated tobacco cells. We tested the effects of a protein kinase inhibitor, staurosporine, on the cell responses. In addition, the states of phosphorylation of total proteins from elicited and nonelicited tobacco cells were compared, using a protocol adapted from Felix et al. (1991).

MATERIALS AND METHODS

Plant Materials and Elicitor

Suspension cultures of tobacco (*Nicotiana tabacum*) cells were grown in the medium of Chandler et al. (1972) as previously described (Milat et al., 1990). Cryptogein was purified according to the method of Ricci et al. (1989) and added to suspension cultures as an aqueous solution.

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Abbreviation: HR, hypersensitive reaction.

Extracellular Alkalization and Potassium Efflux

Cells from cultures in log-phase growth were collected by filtration, washed, and resuspended (0.1 g fresh weight/mL) in 175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, and 2.0 mM Mes buffer adjusted to pH 5.75 with NaOH (Keppler and Baker, 1989). Aliquots of 20 mL were transferred to 50-mL Erlenmeyer flasks and preincubated for 2 h on an orbital shaker (150 rpm) at 25°C.

Mes buffer was titrated, and its buffering capacity was 20.6 μeq H^+/pH unit in the pH range 5.6 to 6.7, which corresponds to the experimental conditions. Extracellular pH and K^+ efflux titrations were monitored for the first 30 min using selective electrodes, and initial velocities were determined. Under these experimental conditions, responses of the K^+ electrode were insensitive to pH changes.

Chemiluminescence

Chemiluminescence was measured using a Beckman LS6000 TA scintillation counter in a single-photon mode. Cells were prepared as previously described and shaken in darkness for 2 h prior to treatment with cryptogein. Samples were transferred to scintillation vials, and luminol was added to a final concentration of 30 μM. Counts were recorded at 3-s intervals and the values obtained at 42 s were reported.

Labeling of Phosphoproteins

Cells prepared in the same manner as for the H^+/K^+ exchange measurements were preincubated for 30 min with cryptogein (5 nM), and then carrier-free [^{32}P]Pi (40 μCi) was added. After a 10-min incubation period, cells were withdrawn by filtration, washed with 5 mL of cold incubation medium, and immediately frozen in liquid nitrogen. Cells were homogenized in 1.2 mL of 10 mM Tris/Mes (pH 7.3) buffer containing 1 mM EDTA, 1 mM PMSF, 1 mM DTT, 1 mM ATP, 250 mM Suc, and 20% glycerol and centrifuged for 15 min at 5,000*g*. Proteins remaining in supernatants (about 1.4 mg/mL, determined by the Bradford method [Bradford, 1976] with BSA as a standard) were precipitated using 10% TCA (1 h at 0°C). Proteins were pelleted (15,000*g* for 3 min) and solubilized in 200 μL of sample buffer by vortex agitation for 45 min (Laemmli, 1970), then incubated for 10 min at 95°C. After centrifugation, 20 μL of the supernatants (containing on average 8 nCi of radioactive P) were subjected to SDS-PAGE (Laemmli, 1970) followed by autoradiography of the dried gel. Molecular mass standards were myosin (205 kD), *Escherichia coli* β-galactosidase (116.2 kD), rabbit muscle phosphorylase b (97.4 kD), BSA (66.2 kD), hen egg white ovalbumin (45 kD), bovine carbonic anhydrase (31 kD), and soybean trypsin inhibitor (21.5 kD).

RESULTS AND DISCUSSION

Physiological Responses of Tobacco Cells to Cryptogein: Sensitivity to Staurosporine

A strong alkalization of the culture medium (Fig. 1A) and a leakage of potassium ions (Fig. 1B) were induced within 5 to 10 min after cryptogein addition to tobacco cell suspensions. These effects reached a plateau within 30 to 40 min and lasted for several hours (data not shown). Staurosporine totally blocked both responses to cryptogein at 1.25 μM (Fig. 1, A and B). Staurosporine alone did not affect pH or K^+ concentrations of the extracellular medium of tobacco cells under these experimental conditions.

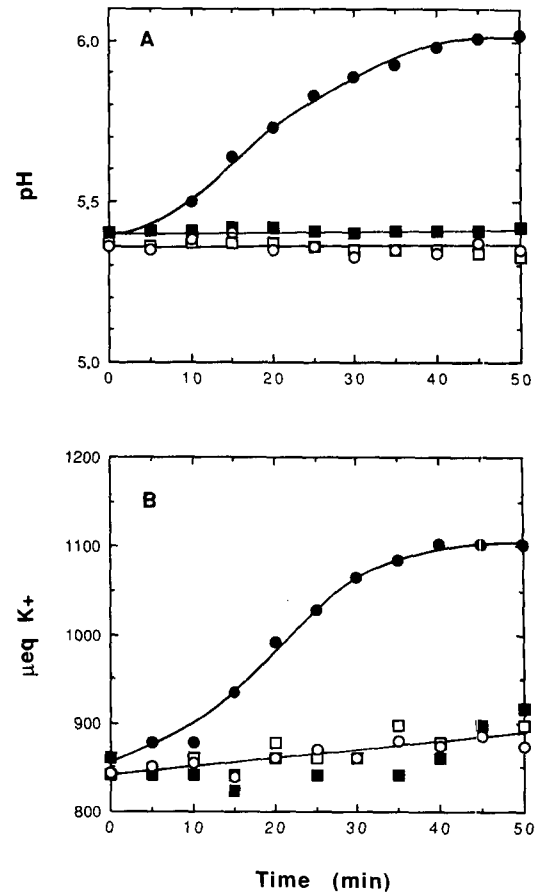

Figure 1. Effect of staurosporine on cryptogein-induced extracellular alkalization (A) and on cryptogein-induced potassium efflux (B) in tobacco cell cultures. ○, Control; □, cells treated with staurosporine (1.25 μM); ●, cells treated with cryptogein (5 nM); ■, cells treated with cryptogein (5 nM) and staurosporine (1.25 μM).

Cryptogein treatment of tobacco cells also induced a rapid, but transient, generation of activated oxygen species, revealed using luminol chemiluminescence (Fig. 2) as described by Schwacke and Hager (1992). The production of activated oxygen species was detected 20 min after the addition of cryptogein, reached a maximum 30 min later, and returned to the control value 90 min after the beginning of the treatment. Control and staurosporine-treated cells produced on average 45 × 10^3 cpm, whereas the response of cryptogein-treated cells reached about 30 × 10^7 cpm. This response was also inhibited by staurosporine (Fig. 2).

In the protein phosphorylation experiments we monitored cryptogein-dependent medium alkalization to confirm that cells were elicited. A dose-response curve showing the effect of staurosporine on the cryptogein-induced medium alkalization (Fig. 1A) is presented in Figure 3. The concentration

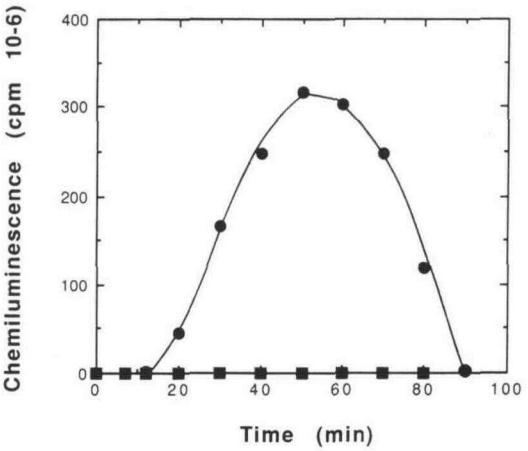

Figure 2. Effect of staurosporine on cryptogein-induced release of active oxygen species from tobacco cells. Cells were treated with cryptogein (5 nM) (●) or with cryptogein (5 nM) and staurosporine (1.25 μM) (■).

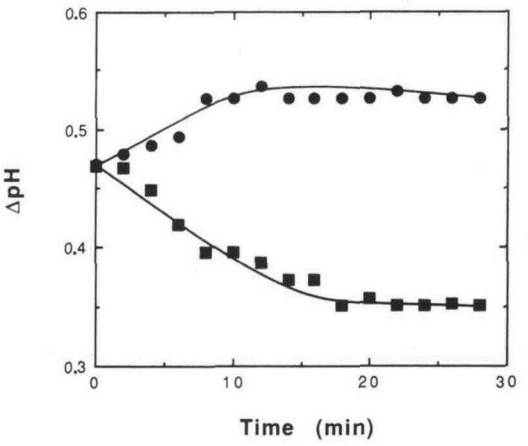

Figure 4. Effect of staurosporine added to tobacco suspension cells during extracellular alkalization induced by cryptogein. Cells were treated with 5 nM cryptogein 30 min before addition of staurosporine (1.25 μM), at zero time. Results are expressed as pH differences between suspensions treated with cryptogein alone (●) or cryptogein + staurosporine (■) and their respective controls without cryptogein.

of staurosporine that inhibited 50% of the effect was about 500 nM.

When staurosporine was added 30 min after cryptogein treatment, i.e. in the stationary phase of the alkalization response, the extracellular pH decreased rapidly, indicating that some proteins must be phosphorylated for the cells to maintain their responses (Fig. 4). For this reason, we also examined the phosphorylation status of total proteins in the time frame 30 to 40 min after cryptogein treatment.

Analysis of Phosphorylated Polypeptides in the Total Protein Fraction

At various times after the addition of ^{32}Pi to cell suspensions, the total proteins were extracted as described in "Materials and Methods." The phosphoprotein labeling increased during the first 5 min and then remained unchanged (data not shown). In further experiments, cell suspensions were allowed to take up ^{32}Pi for 10 min. Figure 5 shows autoradiography of an SDS-PAGE gel of total proteins. Similar results were obtained in three different experiments. Several phosphoproteins appeared in control cells (lane 1). Identical polypeptides were phosphorylated with an equal intensity in

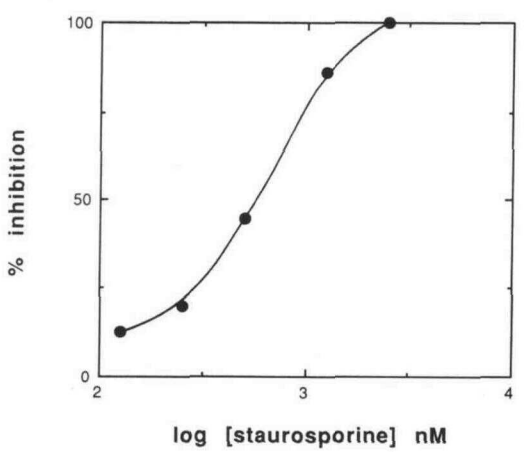

Figure 3. Dose-response curve for staurosporine on medium alkalization in tobacco cell cultures. Cells were simultaneously treated with cryptogein (5 nM) and with various staurosporine concentrations (0.125–2.5 μM). Results were expressed as a percentage of the initial rate of medium alkalization (4.8 neq OH^- min^{-1} mg^{-1} fresh weight).

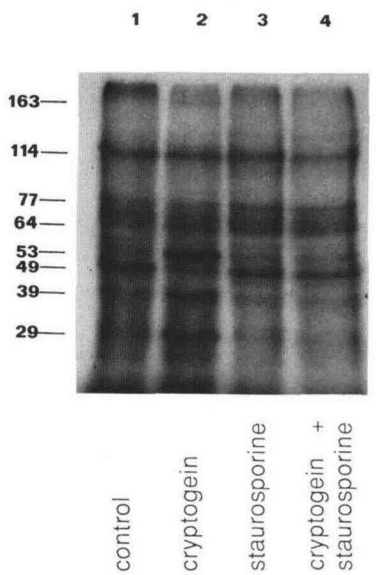

Figure 5. SDS-PAGE of total protein extracts from tobacco cells labeled in vivo with ^{32}Pi for 10 min. Lane 1, Control; lane 2, cryptogein (5 nM); lane 3, staurosporine (1.25 μM); lane 4, cryptogein (5 nM) + staurosporine (1.25 μM). Cells were preincubated for 30 min as mentioned above, then carrier-free ^{32}Pi was added.

staurosporine-treated cells (lane 3). However, cryptogein-induced high-level labeling of a 53-kD polypeptide and, to a lesser extent, 39- and 29-kD polypeptides (lane 2). Two very fine bands appeared at 163 and 77 kD, and labeling of the 49-kD band decreased (lane 2). All of these cryptogein-induced changes in polypeptides disappeared in the presence of staurosporine (lane 4).

Our results indicate that staurosporine, a protein kinase inhibitor, blocks early responses of tobacco cells to cryptogein, i.e. extracellular alkalization, potassium efflux, and production of activated oxygen species. Moreover, cryptogein treatment caused changes in polypeptide phosphorylation that were staurosporine sensitive. In particular, cryptogein induced strong labeling of a 53-kD polypeptide. Taken as a whole, these data suggest that protein kinases may play an essential role in the mechanism of signal transduction leading to an HR. The phosphorylated polypeptides in the response to cryptogein treatment could be protein kinase substrates, protein kinases themselves, or both. Unfortunately, these data do not distinguish between increased phosphorylation and decreased dephosphorylation of proteins due to cryptogein. Under steady-state conditions, the phosphorylation status of any polypeptide depends on the balance of kinase and phosphatase activities that act on it. Nevertheless, decreased phosphorylation in the presence of staurosporine suggests that cryptogein stimulates kinase activity, but further experiments looking at the period just before and during the stationary phase of the response to cryptogein are needed. Other elicitors of different chemical families, i.e. polygalacturonates, have also been reported to enhance membrane polypeptide phosphorylation (Farmer et al., 1991). Thus, protein phosphorylation could be a general mechanism implicated in the activation of plant defense reactions.

A common model for signal recognition and transduction involves a receptor localized on the cell membrane that specifically binds the ligand. This model may be applicable to elicitors because a high-affinity binding protein for the hepta-β-glucoside phytoalexin elicitor in soybean has been characterized (Yoshikawa et al., 1983; Schmidt and Ebel, 1987; Cosio et al., 1988). Cryptogein recognition by tobacco cells may also point toward a membrane receptor because it has been shown that this proteinaceous elicitor specifically binds to tobacco cells (Blein et al., 1991). The aim of our work now is to characterize the putative cryptogein receptor and to identify the different reactions that follow elicitor recognition and lead to the observed physiological responses.

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