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Involvement of Free Calcium in Action of Cryptogein, a Proteinaceous Elicitor of Hypersensitive Reaction in Tobacco Cells[1]

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Treatment of suspension-cultured tobacco (*Nicotiana tabacum* var Xanthi) cells with cryptogein, a proteinaceous elicitor from *Phytophthora cryptogea*, induced a great stimulation of Ca^{2+} influx within the first minutes. Ca^{2+} influx is essential for the initiation of cryptogein-induced responses, since ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid or La^{3+}, which block Ca^{2+} entrance, suppress cryptogein-induced responses such as extracellular alkalinization, active oxygen species, and phytoalexin production. Moreover, once initiated, these responses require sustained Ca^{2+} influx within the 1st h. A Ca^{2+} ionophore (A23187) was able to trigger an extracellular alkalinization but not the formation of active oxygen species and phytoalexins, even in the presence of cryptogein. Staurosporine, a protein kinase inhibitor that was recently reported to suppress cryptogein-induced responses (M.-P. Viard, F. Martin, A. Pugin, P. Ricci, J.-P. Blein [1994] Plant Physiol 104: 1245–1249), inhibited Ca^{2+} influx induced by cryptogein in a dose-dependent manner. These results suggest that protein phosphorylation followed by Ca^{2+} influx might be involved in the initial steps of cryptogein signal transduction.

In plant cells, all of the elements of a Ca^{2+}-based transduction system are found and may function in a manner similar to that of animal cells (Trewavas and Gilroy, 1991). As in animal cells, the cytosolic free Ca^{2+} concentration is low (less than 200 nM) and is tightly controlled by coordinating passive fluxes and active transport across the plasma membrane and organellar membranes such as the ER or tonoplast. Calmodulin, Ca^{2+}-dependent protein kinases, and Ca^{2+}-stimulated phospholipases commonly exist in plants, indicating that changes in cytosolic free Ca^{2+} are likely to have profound effects on cellular functions (Trewavas and Gilroy, 1991; Bush, 1993). Rises in the cytoplasmic Ca^{2+} concentration of higher plants have been implicated in the response to many environmental, hormonal, and pathogenic signals (Leonard and Hepler, 1990). In several plant cell-elicitor systems, some evidence has been obtained that the activation of defense responses depends on the presence of extracellular Ca^{2+} and that a transient increase in the cytoplasmic Ca^{2+} concentration may be a common component of transmembrane signaling (for review, see Ebel and Cosio, 1994). Indeed, results of experiments with Ca^{2+} channel blockers suggest that the influx of Ca^{2+} is a critical event in elicitor-induced signal transduction. In addition, recent results show that mechanisms of phosphorylation/dephosphorylation may participate in signal transduction leading to plant defense responses (Dietrich et al., 1990; Felix et al., 1991, 1994; Viard et al., 1994).

For the past few years, we have been studying the multiple responses of tobacco (*Nicotiana tabacum*) upon treatment with the elicitor cryptogein, a basic holoprotein produced by *Phytophthora cryptogea*. This elicitor causes HR-like necrosis in tobacco plants and also protects the plant against invasion by the pathogen *Phytophthora parasitica* var *nicotianae* (Ricci et al., 1989). Cryptogein rapidly induces an alkalinization of the extracellular medium of tobacco cells, a leakage of potassium (Blein et al., 1991), an acidification of the cytoplasm (A. Pugin, unpublished data), and a transient production of AOS (Bottin et al., 1994). Later, ethylene and phytoalexins accumulate after a lag-phase of 1 and 6 h, respectively (Milat et al., 1991). All of these physiological responses were blocked by staurosporine, a known protein kinase inhibitor (Viard et al., 1994). In the present study, we show that cryptogein induces a fast and large influx of Ca^{2+}, which took place prior to the previously reported effects of cryptogein. Our results indicate that Ca^{2+} influx is essential for induction of the elicitor signal and may represent one of the first events observed in tobacco cells.

MATERIALS AND METHODS

Chemicals and Radiochemical

$^{45}CaCl_2$ (0.92 GBq mg^{-1}) was from Amersham. Verapamil, nifedipine, ionophore A23187, and staurosporine were purchased from Sigma and were added to cell suspensions from concentrated stock solutions in DMSO. Final DMSO concentrations did not exceed 0.5% (v/v). Equivalent volumes of DMSO were added to controls.

Plant Material and Elicitor

Tobacco (*Nicotiana tabacum* var Xanthi) cells were grown in liquid medium as previously described (Milat et al.,

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Abbreviations: AOS, active oxygen species; HR, hypersensitive reaction.

1990). Cryptogein was purified according to the method of Ricci et al. (1989).

Elicitor Treatment

Cells from cultures in exponential-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 mM Mes buffer adjusted to pH 5.75 with KOH (Keppler and Baker, 1989). After a 2-h incubation, tobacco cells were treated with 1 to 200 nM cryptogein in aqueous solution. The control tobacco cells were incubated under the same conditions without cryptogein.

Lanthanum, staurosporine, and EGTA were added to cell suspensions 30 s before addition of cryptogein. Verapamil and nifedipine were added 30 s or 1 h before addition of cryptogein. Cells were simultaneously treated with ionophore A23187 in the presence or in the absence of cryptogein.

Ca^{2+} Influx Measurements

Ca^{2+} uptake measurements were carried out by addition of $^{45}Ca^{2+}$ (0.033 MBq/g fresh weight of cells) 5 min before the treatment with cryptogein. After various periods of treatment (0–90 min), duplicate samples of 3 mL were withdrawn by filtration and washed once for 1 min and twice for 20 s on GF/A glass microfiber filters (Whatman) with 10 mL of ice-cold 2 mM $LaCl_3$ in assay medium without Ca^{2+} to remove extracellular $^{45}Ca^{2+}$. Cells were scraped from the filters, placed in scintillation vials, and weighed. Ten milliliters of Ready Safe cocktail (Beckman) were added to the vials, and the vials were gently shaken overnight before counting in a Beckman LS 600 TA scintillation counter.

In a second step, Ca^{2+} net uptake was measured by flame photometry (AA 300, Varian, Melbourne, Australia). The experiments were carried out as described above without $^{45}Ca^{2+}$. The decrease in extracellular Ca^{2+} was measured in the assay medium after filtration.

Determination of Extracellular pH Changes, AOS, and Phytoalexin Production

Extracellular pH and AOS production were measured, at intervals, in the medium of suspension-cultured tobacco cells. AOS were measured by chemiluminescence of luminol using a luminometer (BCL book): a 250-μL aliquot of the medium was added to 50 μL of 3 mM luminol and 350 μL of assay buffer (175 mM mannitol, 0.5 mM $CaCl_2$, 0.5 mM K_2SO_4, 10 mM Mes, pH 6.5). Values obtained at 40 s were reported. Phytoalexins were extracted from the medium with dichloromethane and analyzed by GC according to Milat et al. (1991).

RESULTS

Ca^{2+} Fluxes after Elicitor Treatment

After a 5-min preincubation with $^{45}Ca^{2+}$, addition of cryptogein resulted in a rapid uptake of $^{45}Ca^{2+}$ within 5 min as illustrated in Figure 1. Cryptogein-induced $^{45}Ca^{2+}$ influx increased continuously and reached a maximum after 60 min (Fig. 1, inset). Comparatively, control cells showed a slight increase in $^{45}Ca^{2+}$ uptake. We verified that cryptogein-induced $^{45}Ca^{2+}$ cellular increase did not result from binding of $^{45}Ca^{2+}$ to cell-wall constituents as a result of the extracellular medium alkalinization. Tobacco cells were washed with La^{3+}-containing medium as described by Reid and Smith (1992) to remove most of the wall-bound $^{45}Ca^{2+}$, and $^{45}Ca^{2+}$ uptake experiments were performed with a constant pH (pH 5.7). Under these conditions, cryptogein induced a similar $^{45}Ca^{2+}$ uptake (not shown). Moreover, addition of calcium ionophore A23187 (5 μM) induced a great increase of $^{45}Ca^{2+}$ uptake (see below).

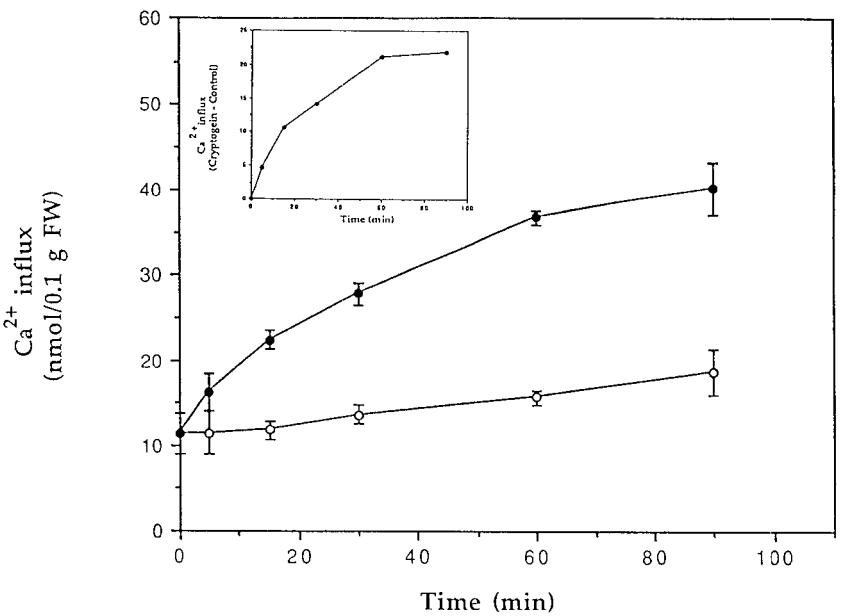

Figure 1. Time course of $^{45}Ca^{2+}$ uptake by tobacco cells after treatment with cryptogein. Cells were preincubated for 5 min in the presence of $^{45}Ca^{2+}$ before addition of cryptogein. Aliquots were withdrawn at the times indicated and analyzed by liquid scintillation counting as described in "Materials and Methods." Results were expressed as an uptake of Ca^{2+} (nmol/0.1 g cell fresh weight) into cells treated with cryptogein (●) or without cryptogein (○). The inset depicts the $^{45}Ca^{2+}$ uptake obtained by subtraction of $^{45}Ca^{2+}$ uptake values in control cells from $^{45}Ca^{2+}$ uptake values in cells treated with cryptogein. The data represent the means of three replicate experiments ± SE. FW, Fresh weight.

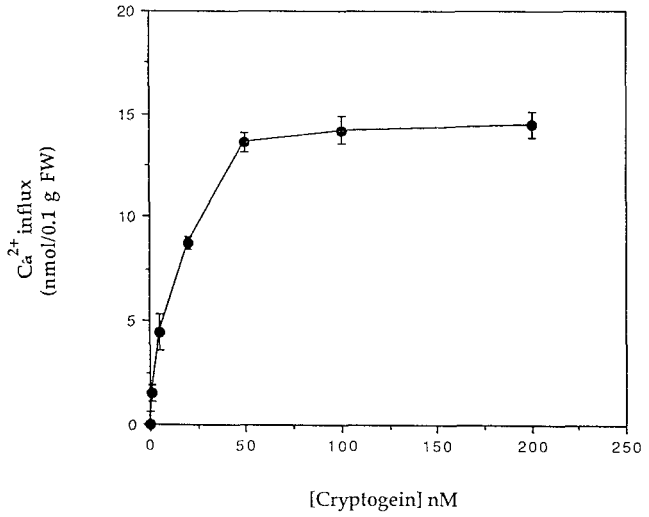

Figure 2. Effect of increasing concentrations of cryptogein on $^{45}Ca^{2+}$ uptake into tobacco cells. The $^{45}Ca^{2+}$ uptake was determined after a 30-min incubation with cryptogein as described in the legend of Figure 1. Values shown were obtained by subtraction of $^{45}Ca^{2+}$ uptake values in control cells from $^{45}Ca^{2+}$ uptake values in cells treated with cryptogein. Results were expressed as means of two replicate experiments ± SE. FW, Fresh weight.

The net Ca^{2+} uptake induced by cryptogein was confirmed using flame photometry to measure the decrease in external medium. After 30 and 60 min of incubation, 14 and 17 nmol of Ca^{2+}, respectively, were taken up from the assay medium by 0.1 g of cell fresh weight.

As shown in Figure 2, $^{45}Ca^{2+}$ uptake was affected by cryptogein in a concentration-dependent manner. Increasing amounts of the elicitor (up to 50 nM) triggered an increase in the $^{45}Ca^{2+}$ intracellular pool, and higher cryptogein concentrations (100 and 200 nM) did not show any additional $^{45}Ca^{2+}$ uptake. This result is well correlated with previous results showing that other cryptogein-induced responses (extracellular alkalinization, AOS, and phytoalexin production) were maximum with a 50 nM concentration of cryptogein.

Effects of Calcium Channel Blockers and EGTA on Cryptogein-Induced Responses

Experiments with EGTA and calcium channel blockers were performed to determine the possible role of Ca^{2+} in transducing the cryptogein signal. When 2 mM EGTA, a preferential Ca^{2+} chelator, was added in the assay medium 30 s before the addition of cryptogein, no $^{45}Ca^{2+}$ uptake was observed. Moreover, extracellular medium alkalinization, AOS, and phytoalexin production did not occur (Table I). The observed inhibition of cryptogein effects was due to calcium chelation, since these effects could be restored by the addition of an excess of calcium.

Tobacco cells were then treated in the presence of La^{3+}, an ion known to block the entrance of Ca^{2+} into cells (Hille, 1992). The results of this series of experiments are depicted in Figure 3. Lanthanum inhibited $^{45}Ca^{2+}$ influx in tobacco cells with a 50% inhibition concentration of 30 to 50 μM, and 75 μM was required for 100% inhibition (Fig. 3A). La^{3+} (100 μM) also inhibited slightly (less than 30%) the basal $^{45}Ca^{2+}$ influx in control cells (not shown). In the same way, La^{3+} inhibited in a dose-dependent manner the extracellular medium alkalinization (Fig. 3B) and the generation of AOS (Fig. 3C). Moreover, AOS production was delayed with increasing La^{3+} concentrations. Lanthanum totally blocked both responses at 75 μM. As with EGTA, La^{3+} was also inhibitory when added 30 min after initiation of the elicitation (Fig. 4, B and C), suggesting that the cryptogein effects, once initiated, required sustained Ca^{2+} influx. In the same way, the synthesis of phytoalexins such as capsidiol was strongly reduced upon addition of 200 μM La^{3+} prior to the treatment with cryptogein (Table I). Thus, a strong correlation was observed between the efficiency for La^{3+} in inhibiting Ca^{2+} influx and in inhibiting the other responses induced by cryptogein.

We tested the effects of verapamil and nifedipine, two organic voltage-gated Ca^{2+} channel antagonists. Verapamil and nifedipine (up to 200 μM) had no effect on either Ca^{2+} influx induced by cryptogein or on associated responses: extracellular pH increase, AOS, and capsidiol production.

Effect of A23187

Because calcium ions were implicated in cryptogein responses, the effects of a calcium ionophore (A23187) were tested on the extracellular medium alkalinization, AOS, and capsidiol production. When added alone or with cryptogein (50 nM) to tobacco cells, A23187 (5 μM) led to an uptake of $^{45}Ca^{2+}$ to a level 4 to 5 times higher than that obtained with cryptogein alone (Fig. 4A).

The addition of A23187 alone to tobacco cells caused an alkalinization of the extracellular medium slightly lower than that induced by cryptogein alone (Fig. 4B). Upon

Table I. *Effects of La^{3+}, EGTA, and A23187 on the cryptogein-induced accumulation of capsidiol in tobacco cell-suspension cultures*

La^{3+} and EGTA were added 30 s prior to cryptogein (Cry) addition (t = 0) and A23187 was added at time t = 0. After 7 h of incubation, the extracellular capsidiol content was determined by GC. Capsidiol content was expressed as μg/10 mL extracellular medium (1 g of cell fresh weight). The values shown are the averages of two replicate experiments ± SD.

Cells	Control	+La^{3+} (0.2 mM)	+EGTA (1 mM)	+A23187 (5 μM)
Control	2.07 ± 1.2	2.51 ± 1.4	n.d.[a]	0.33 ± 0.25
Treated (Cry 50 nM)	21.8 ± 3.1	2.2 ± 2.0	0.15 ± 0.1	2.7 ± 0.8

[a] n.d., Not detected.

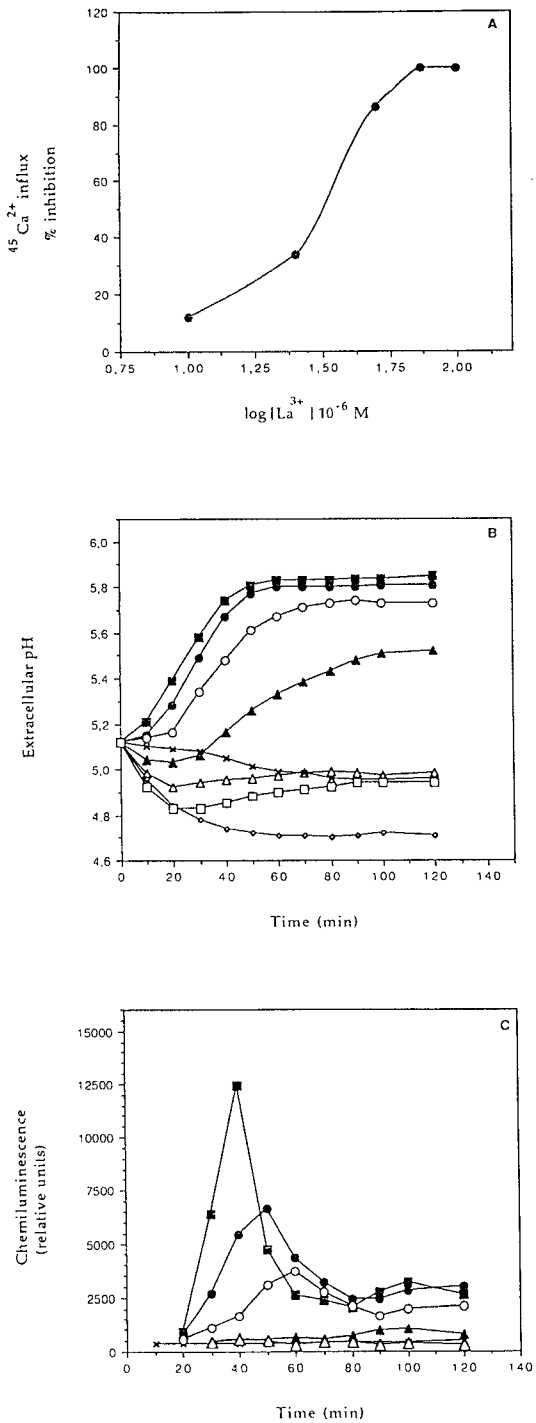

Figure 3. Effects of the Ca^{2+} channel blocker La^{3+} on $^{45}Ca^{2+}$ uptake (A), alkalinization of extracellular pH (B), and AOS production (C) induced by cryptogein. La^{3+} (10–100 μM) was added to cell suspensions 30 s before addition of cryptogein. The dose-response curve for La^{3+} effects on $^{45}Ca^{2+}$ uptake (A) was expressed as a percentage of the $^{45}Ca^{2+}$ uptake after a 30-min incubation with cryptogein. The effect of La^{3+} on cryptogein-induced extracellular alkalinization (B) was determined using a pH electrode. Control cells, (×); cells treated with cryptogein 50 nM (■); with cryptogein 50 nM and La^{3+} 10 μM (●), and La^{3+} 25 μM (○), and La^{3+} 50 μM (▲), and La^{3+} 75 μM (△), and La^{3+} 100 μM (□); control cells treated with La^{3+} 75 μM (◇). Effect of La^{3+} on cryptogein-induced release of AOS (C) was determined by chemiluminescence of luminol. Results were expressed as relative luminescence units. Control cells, (×); cells treated with cryptogein 50 nM (■); with cryptogein 50 nM and La^{3+} 10 μM (●), and La^{3+} 25 μM (○), and La^{3+} 50 μM (▲), and La^{3+} 75 μM (△). The data are representative of three independent experiments.

treatment with both the ionophore and the elicitor, extracellular pH increase was the same compared to that observed with cryptogein alone. The extracellular pH increase induced by A23187 was dose dependent (0.025–10 μM) and attained a maximal value at 5 μM (not shown). In an EGTA-containing and therefore calcium-depleted assay medium, A23187 did not induce any extracellular alkalinization.

As shown in Figure 4C, the addition of ionophore alone did not induce any AOS formation. Treatment of tobacco cells with both A23187 and cryptogein caused a slight delay in AOS production compared to the oxidative burst observed with the elicitor alone (Fig. 4C).

The addition of ionophore alone or with cryptogein did not induce any significant capsidiol production (Table I).

Inhibition by Staurosporine

Staurosporine, a known protein kinase inhibitor, has been previously shown to block cryptogein-induced alkalinization of the extracellular medium of tobacco cells, efflux of potassium, and oxidative burst, and prevented cryptogein-induced changes in the pattern of protein phosphorylation (Viard et al., 1994). A dose-response curve showing the effect of staurosporine on $^{45}Ca^{2+}$ uptake of tobacco cells is presented in Figure 5. Staurosporine slightly enhanced $^{45}Ca^{2+}$ uptake in control cells. Cryptogein-induced $^{45}Ca^{2+}$ uptake was inhibited in a dose-dependent manner. A staurosporine concentration of 1.25 μM was required to totally inhibit the $^{45}Ca^{2+}$ uptake induced by this elicitor. This result is consistent with a previous report showing that the cryptogein-induced responses described above were totally blocked with 1.25 μM staurosporine (Viard et al., 1994).

DISCUSSION

The data presented strongly show that Ca^{2+} influx in tobacco cells is induced in a dose-dependent manner by cryptogein within the first minutes and that the responses, once initiated, require sustained Ca^{2+} influx. In spite of several reports indicating that the activation of defense responses depends on the presence of extracellular Ca^{2+} (for review, see Ebel and Cosio, 1994), there are few examples of direct measurements of Ca^{2+} influx. Atkinson et al. (1990) have reported increased $^{45}Ca^{2+}$ uptake during the HR of tobacco cells after bacterial infection. Conrath et al. (1991) and Nürnburger et al. (1994) also showed elicitor-induced Ca^{2+} uptake in parsley cells using $^{45}Ca^{2+}$ as a tracer. Mathieu et al. (1991) reported that addition of oligogalacturonides to tobacco cells resulted in a decrease of the external $^{45}Ca^{2+}$ concentration. In this type of experi-

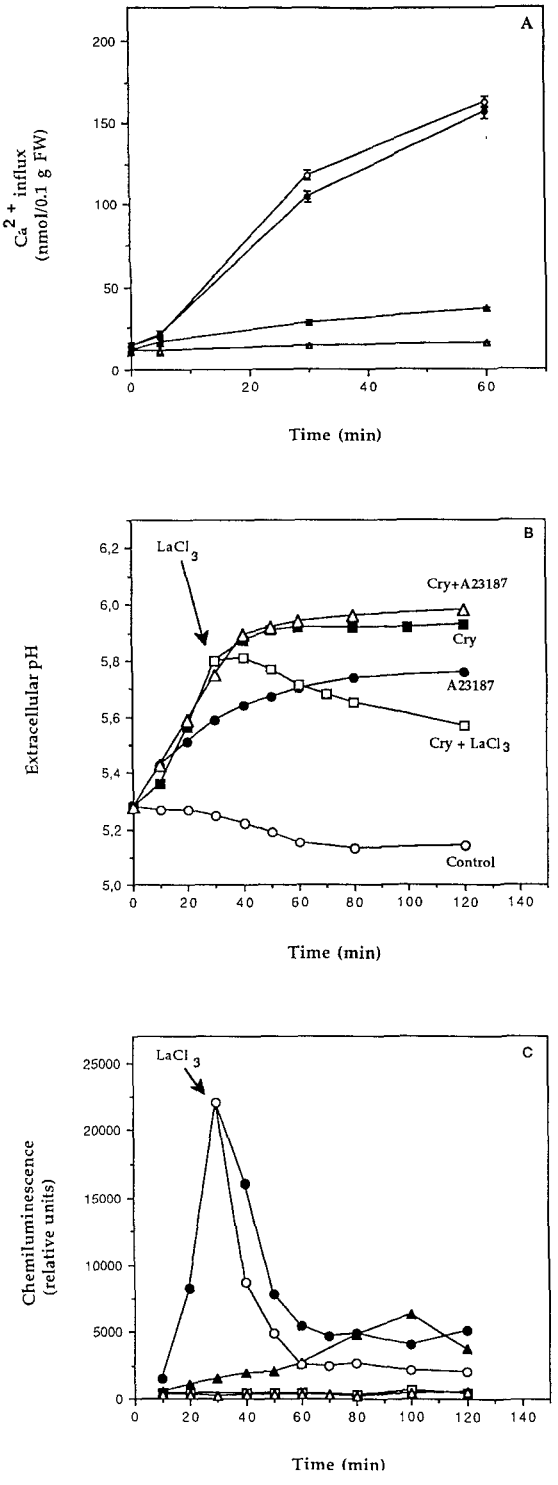

Figure 4. Effect of a calcium ionophore (A23187) on extracellular pH changes (B) and AOS production (C). Cells were simultaneously treated with A23187 (5 μM) in the presence or in the absence of cryptogein. Time course of $^{45}Ca^{2+}$ uptake by tobacco cells after treatment with A23187 (A). Cells were preincubated for 5 min in the presence of $^{45}Ca^{2+}$ before addition of A23187. Aliquots were withdrawn at the times indicated and analyzed by liquid scintillation counting as described in "Materials and Methods." Results were expressed as an uptake of Ca^{2+} (nmol/0.1 g cell fresh weight) into cells treated with cryptogein (●) or without cryptogein (○), control cells without A23187 (△), and cryptogein-treated cells without A23187 (▲). Effect of A23187 on extracellular pH (B) was determined using a pH electrode. La^{3+} was added at time 30 min (arrow). Effect of A23187 on AOS formation (C) was monitored by chemiluminescence of luminol and results were expressed as relative luminescence units. Control cells without A23187 (△) or with A23187 (□); 50 nM cryptogein-treated cells without A23187 (●) or with A23187 (▲); La^{3+} was added at time 30 min (arrow) (○). The data are representative of three independent experiments. FW, Fresh weight.

ment, cell-wall-bound calcium complicates the interpretation of data (Mac Robbie, 1989; Kauss et al., 1992). Nevertheless, under our conditions, the increase in intracellular $^{45}Ca^{2+}$ might not result from binding of Ca^{2+} to cell-wall constituents. Fixation of free Ca^{2+} by cell-wall constituents has been overcome using protoplasts (Bach et al., 1993), but such experiments must show that protoplasts exhibit the same response to the elicitor as the respective suspension-cultured cells. The $^{45}Ca^{2+}$ influx measured after a short 5-min treatment and the decrease in the total external calcium measured using flame photometry fit well with a net uptake of Ca^{2+} induced by cryptogein. Moreover, the magnitude of this external Ca^{2+} decrease is in close correlation with the cryptogein-induced Ca^{2+} influx observed with $^{45}Ca^{2+}$ uptake experiments.

Our results indicate that, to our knowledge, protein phosphorylation (Viard et al., 1994) and Ca^{2+} influx are the earliest (indeed the first) cryptogein-induced events observed in our system. Moreover, these events are essential for the initiation and the maintenance of the previously described effects of cryptogein: extracellular alkalinization, AOS, and capsidiol production. At first, a Ca^{2+}-deprived medium inhibits the cryptogein-induced events. Moreover, La^{3+}, which is thought to remain outside cells and block the entry of Ca^{2+}, inhibited Ca^{2+} influx in a dose-dependent manner and prevented the initiation and maintenance of cryptogein-induced events. These results are consistent with previous reports indicating that La^{3+} in the same range of concentration could prevent K^+/H^+ response in tobacco cells elicited by either *Pseudomonas syringae* (Atkinson at al., 1990) or *Erwinia amylovora* Harpin (Yang He et al., 1994). Similar to results in our system, Baker et al. (1993) have reported that La^{3+} blocked AOS production when added simultaneously with the elicitor and that the addition of La^{3+}, 10 min after elicitor treatment (30 min under our conditions), had a slight inhibitor effect on AOS generation. In the same way, La^{3+} is an efficient inhibitor of phytoalexin accumulation in tobacco cells elicited by cryptogein or cellulase (Preisig and Moreau, 1994) or in potato tuber tissue elicited by arachidonic acid (Zook et al., 1987). Since La^{3+} is known to block calcium channels in animal and plant cells (Hille, 1992), the La^{3+}-inhibited Ca^{2+} influx reported here may be mediated by calcium channels. Verapamil and nifedipine, which block voltage-dependent calcium channels in plant cells (Graziana et al., 1988; Schumaker and Gizinski, 1993; Pineiros and Tester, 1995), had no effect on Ca^{2+} influx, extracellular alkalinization, and AOS production induced by cryptogein. Taken

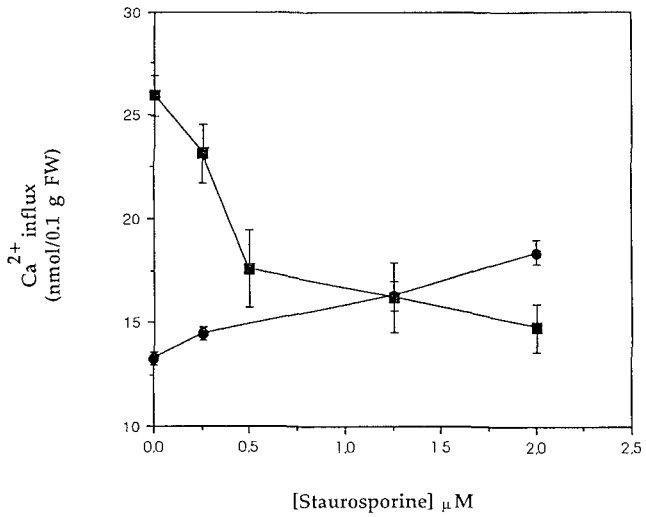

Figure 5. Dose-response curve for staurosporine on the inhibition of $^{45}Ca^{2+}$ uptake in tobacco cells. Various staurosporine concentrations (0.25–2 μM) were added 30 s prior to 50 nM cryptogein treatment (■) or in the absence of cryptogein (●). $^{45}Ca^{2+}$ uptake was determined after a 30-min incubation and the results were expressed as means of three replicate experiments ± SE.

together, these results suggest that if calcium channels are involved in cryptogein-induced calcium influx, they are not of the voltage-gated type but could be of the ligand-dependent type. However, our results do not prove direct calcium channel involvement, and studies using calcium channel antagonists such as verapamil in plant physiological responses should be regarded with caution (Thomine et al., 1994). Patch-clamp studies are required to verify this hypothesis.

According to the hypothesis that Ca^{2+} influx has been identified as one of the signaling-pathway-initiating cryptogein responses, we tested the effects of the ionophore A23187, which is able to cause the elevation of intracellular Ca^{2+} in tobacco cells. Without the application of cryptogein, A23187 was able to trigger an extracellular alkalinization, but in no way the formation of AOS and phytoalexins. The alkalinization of the extracellular medium induced by A23187 was slightly lower than that induced by cryptogein. But, because A23187 is an antiporter H^+/Ca^{2+} (Shumaker and Gizinski, 1993), the alkalinization of the extracellular medium induced by A23187-mediated Ca^{2+} influx is certainly underestimated.

On the other hand, addition of A23187 simultaneously with the elicitor resulted in only a small and delayed production of AOS compared to addition of cryptogein alone, and did not induce any significant phytoalexin production. These results are in contrast to (a) a small AOS production and a usual AOS production induced by A23187 alone and by both A23187 and a fungal elicitor, respectively, in cultured spruce cells (Schwacke and Hager, 1992), and (b) phytoalexin formation induced by A23187 with or without elicitor in carrot cells (Kurosaki et al., 1987). Our results suggest also that there is a strong correlation between Ca^{2+} influx and extracellular alkalinization. This effect could, among other mechanisms, imply an inactivation of the plasma membrane H^+-ATPase by free cytosolic calcium or mediated by Ca^{2+}-activated protein kinase(s). Indeed, the tobacco plasma membrane H^+-ATPase is inhibited by low Ca^{2+} concentration (not shown), and the autoinhibitory C-terminal domain of the H^+-ATPase can be phosphorylated by a Ca^{2+}- and phospholipid-dependent kinase inducing inactivation of this enzyme (Serrano, 1989; Shaller et al., 1992). Inversely, the mechanism for extracellular acidification induced by fusicoccin or a fungal elicitor may involve the stimulation of the plasma membrane H^+-ATPase mediated by the release of the C terminus or by dephosphorylation of this domain (Johansson et al., 1993; Vera-Estrella et al., 1994). Results concerning AOS production suggest that the oxidative burst is induced by a specific Ca^{2+} influx, since Ca^{2+} influx induced by A23187 alone or both A23187 and cryptogein abolished the role of Ca^{2+} as a second messenger in signal transduction. However, the involvement of additional second messengers or signal pathways in the initiation of AOS or phytoalexin production cannot be dismissed.

Recently, rapid changes in protein phosphorylation have been reported to be involved in the transduction process of some elicitor signals (Dietrich et al., 1990; Felix et al., 1991). These changes in the phosphorylation status of specific proteins as well as the early biochemical responses to elicitors can be blocked by inhibitors of protein kinases such as staurosporine and K-252a (Conrath et al., 1991; Felix et al., 1991; Schwacke and Hager, 1992; Viard et al., 1994). Moreover, Felix et al. (1994) reported that calyculin A, an inhibitor of protein phosphatases, can mimic elicitor action in several aspects. Since early cryptogein-induced responses in tobacco cells were fully inhibited by staurosporine (Viard et al., 1994), we investigated the effect of this inhibitor on Ca^{2+} influx. Our results show that staurosporine inhibits the Ca^{2+} influx induced by cryptogein in a dose-dependent manner and in the same range of concentration that was previously observed for other cryptogein-induced responses. This result is consistent with that obtained in parsley cells elicited by a fungal elicitor (Conrath et al., 1991). Taken as a whole, these results indicate that the mechanism of phosphorylation/dephosphorylation may play an essential role in the first events of the transduction signal and suggest that cryptogein action could be based on activation of a protein kinase (as well as inhibition of a protein phosphatase) and/or autophosphorylation especially leading to a Ca^{2+} influx.

The aim of our work is now to characterize the magnitude of the HR-associated Ca^{2+} influx. With regard to calcium as a second messenger, a transient change in cytosolic free Ca^{2+} concentration could be expected if one considers that cells tightly regulate the cytosolic level of Ca^{2+} through buffering Ca^{2+}-binding proteins and increased transport into the vacuole, other organelles, or the apoplast (Clapham, 1995). Moreover, the massive influx of Ca^{2+} that leads to an increase in Ca^{2+} concentration of approximately 250 μM in elicited tobacco cells within 30 min suggests that the entry of Ca^{2+} could induce plasma membrane depolarization. This effect could be an addi-

tional event involved in the cryptogein signal transduction. We now need to measure the effect of cryptogein on the plasma membrane potential of tobacco cells related to this Ca^{2+} influx. Patch-clamp studies are now in progress to confirm the involvement of calcium channels in cryptogein-induced Ca^{2+} influx. These studies will also be directed toward the identification of the type of channel involved (ligand-dependent or voltage-dependent) and its mode of regulation.

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