

Comparison of Binding Properties and Early Biological Effects of Elicitins in Tobacco Cells¹

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Elicitins are a family of small proteins secreted by *Phytophthora* species that have a high degree of homology and elicit defense reactions in tobacco (*Nicotiana tabacum*). They display acidic or basic characteristics, the acidic elicitors being less efficient in inducing plant necrosis. In this study we compared the binding properties of four elicitors (two basic and two acidic) and early-induced signal transduction events (Ca^{2+} influx, extracellular medium alkalization, and active oxygen species production). The affinity for tobacco plasma membrane-binding sites and the number of binding sites were similar for all four elicitors. Furthermore, elicitors compete with one another for binding sites, suggesting that they interact with the same receptor. The four elicitors induced Ca^{2+} influx, extracellular medium alkalization, and the production of active oxygen species in tobacco cell suspensions, but the intensity and kinetics of these effects were different from one elicitor to another. As a general observation the concentrations that induce similar levels of biological activities were lower for basic elicitors (with the exception of cinnamomin-induced Ca^{2+} uptake). The qualitative similarity of early events induced by elicitors indicates a common transduction scheme, whereas fine signal transduction tuning is different in each elicitor.

Elicitins are a family of proteins excreted by *Phytophthora* spp., a group of widespread and highly damaging pathogenic fungi (Ricci et al., 1993; Yu, 1995). These proteins have the property to elicit defense responses in tobacco (*Nicotiana tabacum*), triggering a hypersensitive response and systemic resistance to further inoculation (Bonnet et al., 1996; Keller et al., 1996). These 98 amino acid proteins show more than 60% sequence identity with each other. They are devoid of posttranslational modifications and possess six invariant Cys residues that account for three disulfide bridges, which were first established in capsicein, an elicitor secreted by *Phytophthora capsici* (Bouaziz et al., 1994).

Circular dichroism experiments performed on four elicitors, cryptogein, cinnamomin, parasiticein, and capsicein, together with capsicein structure analysis by NMR, led to

the conclusion that the elicitor folding was mainly α -helical (Nespoulous et al., 1992; Bouaziz et al., 1994). A recent study (Boissy et al., 1996) determined the crystal structure of cryptogein, confirming the existence of three disulfide bridges. These authors proposed a globular structure composed of six α -helices and a beak-like motif built from invariant residues, forming an antiparallel, two-stranded β -sheet and an Ω -loop. However, beyond these general structural features, elicitors have long been classified into two groups according to their differences in net charge, the acidic or α -elicitors (pI between 3 and 5) and the basic or β -elicitors (pI between 7 and 9).

This classification correlates with the biological properties of elicitors in tobacco plants. The amount of basic elicitors required to induce leaf necrosis or protection against *Phytophthora parasitica* var *nicotianae* is 50- to 100-fold lower than for acidic elicitors (Ricci et al., 1989; Nespoulous et al., 1992; Pernollet et al., 1993; Bonnet et al., 1996). Necrotic activity was not the result of different migration rates or elicitor accumulation, since ¹²⁵I-labeled elicitors (the basic cryptogein and the acidic capsicein) showed the same distribution within the plant (Devergne et al., 1992; Zanetti et al., 1992). Instead, this activity should depend on the high number of Lys residues present in basic elicitors, which contributed to their net charge. Particularly the mutation of Lys-13 resulted in a decrease in necrotic activity (O'Donohue et al., 1995).

The difference in biological activity between α - and β -elicitors, which is associated with their structural properties, might be correlated with one or several steps of the signal transduction pathway induced in the target cells. It is generally assumed that a putative receptor on the plasma membrane acts as the first component of the transduction pathway, and then secondary messengers, membrane proteins, or cytosolic proteins such as protein kinases transduce the signal to activate plant-defense responses (Staskawicz et al., 1995). Perception of elicitors by the target cell and transduction of this signal should direct the physiological effects observed at the plant level, emphasizing the importance of studying the initial steps of the transduction pathway.

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Abbreviations: AOS, active oxygen species; IC_{50} , half-maximal inhibitor concentration.

Among elicitors cryptogein has been extensively studied, and early steps of its interaction with tobacco cells have been defined. Cryptogein high-affinity binding sites characterized on tobacco plasma membrane preparations had properties consistent with receptor sites (Wendehenne et al., 1995). Analysis of early events induced by this elicitor were performed on tobacco cell suspensions. Ca^{2+} influx (Tavernier et al., 1995b), modifications of ion fluxes (H^+ , K^+ , and Cl^- ; Blein et al., 1991; Pugin et al., 1997), depolarization of the plasma membrane (Pugin et al., 1997), production of AOS (Bottin et al., 1994), protein phosphorylation (Viard et al., 1994), cytosol acidification (Barbier-Brygoo et al., 1997; Pugin et al., 1997), and changes in gene expression (Suty et al., 1996; Petitot et al., 1997) were rapidly monitored after a few minutes of cryptogein treatment. Later, changes in lipid composition (Tavernier et al., 1995a) and the production of phytoalexin and ethylene were measured (Milat et al., 1991).

Protein phosphorylation and Ca^{2+} influx were the earliest effects monitored after cryptogein-tobacco cell interaction. Similar early effects were reported in other elicitor-plant cell interactions. Tomato cells treated with a yeast-extract-derived elicitor, a fungal xylanase elicitor, or chitin fragments produced an increase in extracellular pH and rapid changes in protein phosphorylation (Felix et al., 1991, 1994; Baureithel et al., 1994). Changes in the permeability of the plasma membrane to different ions (H^+ , Ca^{2+} , K^+ , and Cl^-) were among the earliest events detectable after treatment of parsley cells with a 42-kD glycoprotein from *Phytophthora megasperma* (Nürnberg et al., 1994) or after treatment of tobacco cells with *Pseudomonas syringae* pv *syringae* (Atkinson et al., 1990). Tobacco cells incubated with oligogalacturonides developed rapid cytoplasmic acidification and extracellular pH alkalinization, which depend on a phosphorylation process (Mathieu et al., 1996a, 1996b). AOS production, which is often correlated with plant defense reactions, is triggered by elicitors from different organisms (Medhy, 1994) and is probably coupled to ion flux modifications (Baker et al., 1993; Tavernier et al., 1995b). In cryptogein-treated tobacco cells, AOS production depends on Ca^{2+} influx. La^{3+} , a Ca^{2+} -channel blocker, inhibited extracellular alkalinization and AOS production (Tavernier et al., 1995b). Although the mechanisms linking ion fluxes and AOS generation to the elicitor-recognition step are not clearly defined, there was a tight and close connection between elicitor perception and physiological events.

In the present work we compared the binding properties of two acidic elicitors (capsicein and parasiticein) and two basic elicitors (cryptogein and cinnamomin) and studied their biological effects on tobacco cell suspensions (Ca^{2+} influx, alkalinization of the extracellular medium, and AOS production). We wanted to determine whether elicitors share different affinities for binding sites on tobacco plasma membranes, whether they have different activities according to their biophysical and structural properties, and whether the binding capacities may direct intensities of the early responses of tobacco cells involved in signal transduction after the recognition step.

MATERIALS AND METHODS

Plant Materials and Elicitors

Tobacco (*Nicotiana tabacum* cv Xanthi) plants were grown in a greenhouse for 60 d. Cell suspensions were cultivated in Chandler's medium (Chandler et al., 1972) on a rotary shaker (150 rpm, 25°C) under continuous light (photon flux rate 30–40 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Cells were maintained in the exponential phase and subcultured 1 d prior to utilization. Elicitors were purified according to the method of Bonnet et al. (1996).

Plasma Membrane Preparation

Purification of the plasma membrane was as previously described by Wendehenne et al. (1995) using the aqueous partitioning procedure of Widell et al. (1982). Tobacco microsomal fractions obtained from leaves (400 g) homogenized in 800 mL of grinding medium (50 mM Tris-Mes, pH 8.0, 0.5 M Suc, 20 mM EDTA, 10 mM DTT, and 1 mM PMSF) were added to an aqueous polymer two-phase system with final concentrations of 6.6% (w/w) dextran (M_r 500,000) and 6.6% (w/w) PEG (M_r 3,350) in a PSK buffer (5 mM phosphate buffer, pH 7.8, 0.3 M Suc, and 3 mM KCl). Plasma membrane-enriched fractions were suspended in 10 mM Tris-Mes, pH 6.5, 250 mM Suc, 1 mM PMSF, 2 mM MgCl_2 , and 20% glycerol and stored at -80°C . Protein content was measured according to the method of Lowry et al. (1951). The low level of contaminants in the plasma membrane preparations was confirmed using marker enzymes (Larsson et al., 1994).

Binding Experiments

Iodination of elicitors was performed as previously described (Wendehenne et al., 1995). One hundred micrograms of elicitor was incubated for 20 min at 20°C in 100 μL of 0.1 M phosphate buffer, pH 7.4, with 185 MBq Na^{125}I (specific activity 625 MBq/ μg iodine; Amersham) and Iodogen (Pierce) as the catalyst. Nonincorporated iodine was removed by gel filtration using a Sephadex G-25 column equilibrated with 50 mM Tris-HCl buffer, pH 7.4. Specific activity of radiolabeled elicitor was about 9.25 TBq/mmol. Binding experiments were carried out as previously described (Wendehenne et al., 1995), except for the binding medium (10 mM Hepes-KOH, pH 7.0, 5 mM MgCl_2 , and 0.1 M Suc). Binding of ^{125}I -elicitors on plasma membrane preparations (50 μg of protein) was carried out on ice for 90 min after the addition of the appropriate concentration of radiolabeled elicitor (and 10 μM unlabeled elicitor in assays for nonspecific binding). Binding of ^{125}I -elicitors was monitored by filtration under a vacuum using GF/B glass-fiber filters (Whatman). These filters were then washed three times with ice-cold binding buffer containing 0.1% BSA. Radioactivity remaining on the filters was measured in 5 mL of Ready-Safe cocktail (Beckman) with a gamma counter (model LS 600 TA, Beckman). Competition experiments were performed using 10^{-11} to 10^{-4} M unlabeled elicitors and 10 nM labeled elicitors.

Elicitor Treatment

Cells were collected during the exponential growth phase and washed by filtration in a suspension buffer containing 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, and 2 mM Hepes adjusted to pH 5.75 with KOH (Keppler and Baker, 1989). Cells were resuspended at 0.1 g fresh weight mL⁻¹ with suspension buffer and equilibrated for 2 h on a rotary shaker (150 rpm, 24°C). Tobacco cells were then used for determination of extracellular pH changes, Ca²⁺ influx measurements, and AOS production after treatment with 5 to 500 nM elicitor in aqueous solution. Control tobacco cells were incubated under the same conditions without elicitors.

Ca²⁺ Influx Measurements

Ca²⁺ influx measurements were performed as previously described (Tavernier et al., 1995b). Five minutes before the treatment with elicitors, cell suspensions were incubated with ⁴⁵Ca²⁺ (0.033 MBq g⁻¹ fresh weight cells; Amersham). After different periods of treatment, duplicate 2-mL aliquots were collected and filtered under a vacuum on GF/A glass-fiber filters, washed once for 1 min with 10 mL of ice-cold cell-suspension buffer containing 2 mM LaCl₃, and then washed twice with 5 mL of the suspension buffer for 20 s. Cells remaining on filters were collected, dried overnight at 70°C, weighed, and placed in scintillation vials with 10 mL of Ready-Safe cocktail. Vials were shaken overnight before counting in a scintillation counter.

Extracellular pH Changes and AOS Production

Extracellular pH changes were measured at 10-min intervals in tobacco cell suspensions. AOS production was determined using chemiluminescence of luminol. Aliquots (250 μL) of cell suspensions were collected and mixed with 300 μL of 10 mM Hepes buffer, pH 6.5, 175 mM mannitol, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, and 50 μL of 0.3 mM luminol. Chemiluminescence measured within a 10-s period with a luminometer (EG&G Wallac, Gaithersburg, MD) was integrated and expressed in nanomoles of H₂O₂ per gram fresh weight of cells.

RESULTS

Binding Characteristics

Each elicitor was labeled with ¹²⁵I with a specific activity of about 9.25 TBq/mmol, which corresponds to the radiolabeling of 12% of the molecules. This treatment did not modify elicitor activities such as alkalization of the extracellular medium or AOS production (data not shown). Moreover, these activities were unaffected when elicitors were derivatized with a least one nonradioactive iodine per molecule (data not shown).

Binding experiments were performed by incubating 50 μg of plasma membrane proteins with increasing concentrations (1–30 nM) of each ¹²⁵I-elicitor (Fig. 1). Nonspecific binding on the plasma membrane was similar for each elicitor, whereas when elicitors were used at 10 nM specific

¹²⁵I-elicitor binding was between 2500 and 3500 cpm, depending on each ¹²⁵I-elicitor specific activity. The saturation curves were hyperbolic, with half-maximal binding concentrations at about 10 nM. Scatchard plots gave straight lines, indicating the presence of a single class of specific binding sites for each elicitor (Fig. 1, insets). The K_d values and the number of binding sites deduced from Scatchard plots are shown in Table I. The K_d of cryptogein (10.3 nM) was similar to that previously obtained in a Tris buffer (K_d = 2 nM; Wendehenne et al., 1995).

The K_d for the other elicitors ranged from 5.8 to 13.5 nM, showing that the four elicitors, independently of their acidic or basic properties, bound with the same affinity to plasma membrane-binding sites. Furthermore, the number of binding sites present on the plasma membrane was similar for each elicitor (Table I). Specific binding as a function of pH and tested at a pH range of 4.0 to 9.0 was optimum at pH 7.0 for each elicitor (Fig. 2). These results suggest that the four elicitors bind to common sites through invariant amino acids. Competition experiments were performed to verify this hypothesis.

Competition of Elicitors for Binding Sites

¹²⁵I-cryptogein (10 nM) was incubated with plasma membrane preparations in the presence of increasing concentrations of unlabeled elicitors or elicitors derivatized with nonradioactive iodine. The corresponding IC₅₀ values are reported in Tables II and III. Results indicated that the four unlabeled elicitors compete with labeled cryptogein (10 nM) for specific binding sites: the IC₅₀ for unlabeled cryptogein, capsicein, or parasiticein was similar. The IC₅₀ for unlabeled cinnamomin was 4 to 5 times higher than unlabeled cryptogein IC₅₀ (Table II). The reverse-competition experiments using the four labeled elicitors (10 nM each) in competition with unlabeled cryptogein gave similar cryptogein IC₅₀ values whatever the elicitor, with only a 2-fold variation (Table II). In this experiment the IC₅₀ values were comparable to those obtained when unlabeled elicitors were competing with ¹²⁵I-cryptogein.

These results showed that elicitor-binding properties were comparable and that elicitors were acting as competitors; however, these IC₅₀ values were higher than the expected values, which should reach the K_d values. This discrepancy could reflect different affinities of iodinated and noniodinated elicitors for binding sites. Therefore, competition experiments were also performed with increasing concentrations of elicitors derivatized with nonradioactive iodine (Fig. 3; Table III). In these conditions the IC₅₀s for unlabeled iodinated elicitors were similar (close to the K_d of the labeled elicitor), suggesting that iodinated elicitors were better competitors for the plasma-binding sites than noniodinated elicitor. Consequently, K_d values should better reflect the iodinated elicitor affinities. However, all elicitors had a similar shifted IC₅₀ value when iodinated; therefore, binding properties are comparable among the four elicitors, all of which bind to the same specific binding sites with a similar affinity.

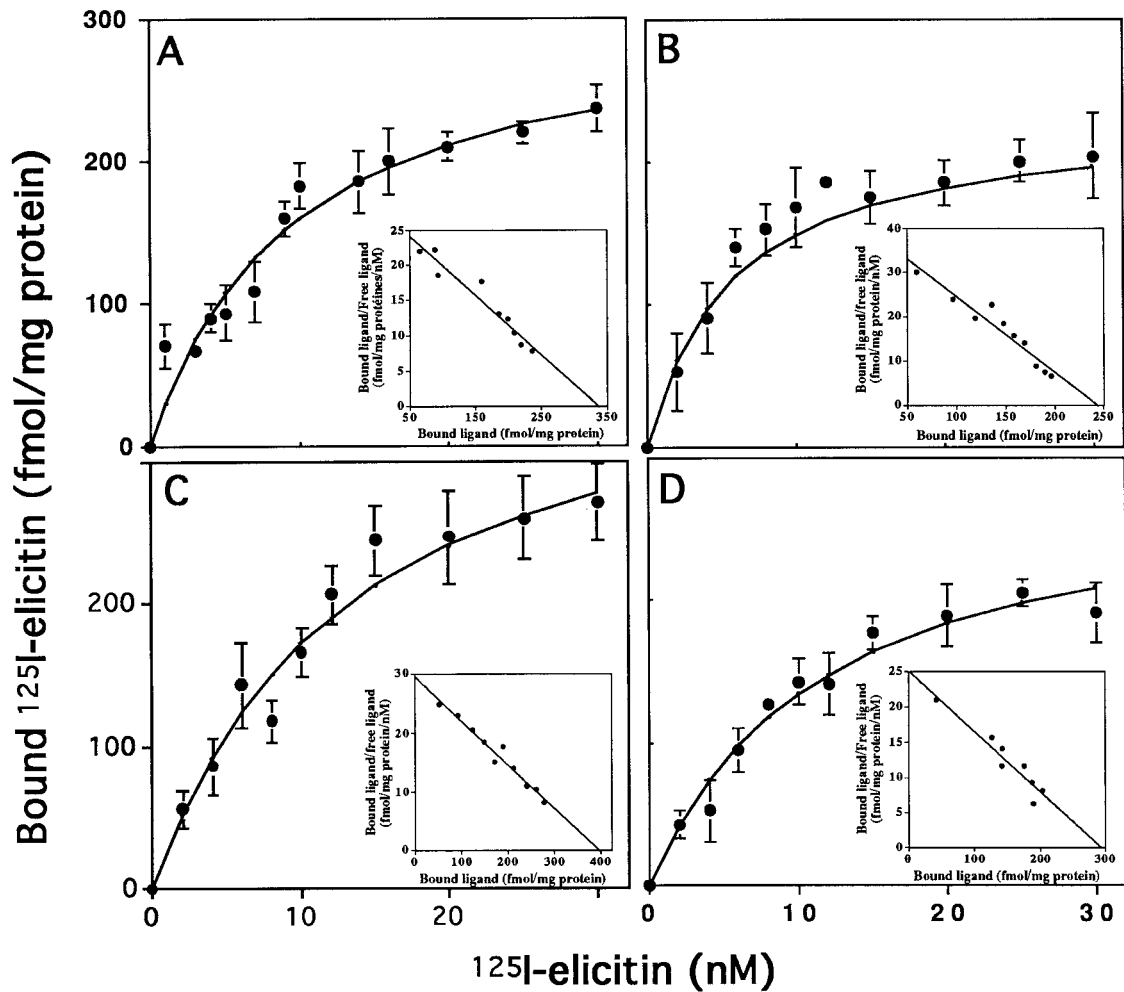


Figure 1. Saturation curves obtained with labeled elicitors and corresponding Scatchard plots (insets). Plasma membrane preparations were incubated with various concentrations of ^{125}I -cryptogein (A), ^{125}I -cinnamomin (B), ^{125}I -capsicein (C), or ^{125}I -parasiticein (D). Nonspecific binding was measured in the presence of a $10\ \mu\text{M}$ concentration of the corresponding nonlabeled elicitor and was subtracted from total binding to give the saturation curves. Experiments were repeated at least three times. Results are means \pm SD.

Extracellular Medium Alkalinization

Cell suspensions were treated with various concentrations of elicitors (5–500 nM), and extracellular pH changes were measured in the cell-suspension medium for 90 min (Fig. 4). The four elicitors induced an extracellular pH increase. However, the kinetics and intensity of this response were specific for each elicitor and depended on their respective concentrations. For example, at a low concentration (10 nM) cryptogein induced a rapid and strong alkalinization of the extracellular medium, reaching the half-maximum pH increase in less than 10 min, whereas no significant or only slight pH changes were measured during the first 10 min with the other elicitors. The extracellular pH stabilized 40 to 60 min following elicitor treatment. However, the pH plateau depended on the elicitor used and gradually decreased in cinnamomin, parasiticein, and capsicein (in this order). When the four elicitors were used at higher concentrations (100 nM) the maximal medium alkalinizations were similar after treatment for 90 min,

although initial rates for alkalinization were $0.085\ \text{pH unit min}^{-1}$ for cryptogein, $0.047\ \text{pH unit min}^{-1}$ for capsicein and parasiticein, and $0.020\ \text{pH unit min}^{-1}$ for cinnamomin.

The lowest elicitor concentrations inducing the highest alkalinization were about 10 nM for cryptogein and 50 nM for cinnamomin, whereas those for the acidic elicitors were about 500 nM. These results show that basic elicitors were at least 10 times more efficient than acidic elicitors in inducing maximal extracellular pH alkalinization.

Ca^{2+} Influx

Using $^{45}\text{Ca}^{2+}$ as a tracer we compared Ca^{2+} uptake induced by the four elicitors at three concentrations: 10, 100, and 500 nM. These assays were performed in the incubation medium used to monitor the alkalinization (low-buffered medium, pH 5.75). At 500 nM the four elicitors induced an uptake of $^{45}\text{Ca}^{2+}$ (Fig. 5A). In the same experiments control cells did not show any $^{45}\text{Ca}^{2+}$ uptake.

Table I. ^{125}I -elicitin binding characteristics in tobacco plasma membranes

Specific binding was obtained by subtracting nonspecific binding from total binding. Data were obtained from Scatchard plots deduced from saturation curves and are the averages \pm SD of three experiments.

^{125}I -Elicitin	K_d nM	Specific Binding Sites fmol mg^{-1} plasma membrane proteins
Cryptogein	10.3 ± 1.8	318 ± 37
Cinnamomin	5.8 ± 2.6	234 ± 25
Capsicein	13.5 ± 3.1	403 ± 60
Parasiticein	10.9 ± 4.4	280 ± 71

Cryptogein was the most efficient elicitor, whereas the other elicitors induced a similar but lower Ca^{2+} uptake. In a second experiment Ca^{2+} influx was measured after treatment for 1 h with increasing concentrations of elicitors. Cryptogein was still the most efficient elicitor whatever the concentration (Fig. 5B); the other elicitors were less efficient in inducing $^{45}\text{Ca}^{2+}$ uptake.

Because at pH 5.75 elicitors showed different relative specific binding, Ca^{2+} uptake induced by elicitors at 100 nM was also measured in a 50 mM Hepes medium buffered at pH 7.0, which corresponds to the optimum pH for the binding of the four elicitors. Under these conditions we obtained results comparable to those obtained at pH 5.75 (low-buffered medium; Fig. 5B), with the exception of cinnamomin, which triggered a lower Ca^{2+} uptake compared with the other elicitors. Therefore, even when used at their optimum binding pH, the elicitors triggered different Ca^{2+} uptakes (Fig. 5C).

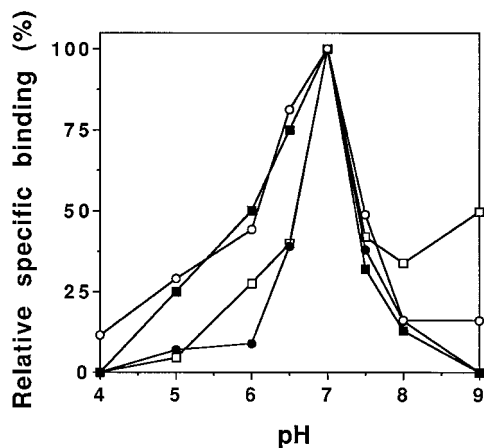


Figure 2. pH dependence of the specific binding of ^{125}I -elicitors on tobacco plasma membrane preparations. Fifty micrograms of plasma membrane proteins was incubated with 10 nM ^{125}I -cryptogein (\circ), ^{125}I -cinnamomin (\bullet), ^{125}I -capsicein (\square), or ^{125}I -parasiticein (\blacksquare) in the following buffers: citrate-KOH (pH 4.0–6.0), Hepes-KOH (pH 6.5–8.0), and Tris-HCl (pH > 8.0). Buffer strength was 10 mM. Specific binding was expressed as a percentage of the maximal specific binding measured for each elicitor. The experiment was repeated three times. Each data point represents the average of triplicate measurements.

Table II. Inhibition of ^{125}I -elicitin specific binding by noniodinated elicitors

IC_{50} values were determined from competition curves. Data are the averages \pm SD of three experiments.

^{125}I -Elicitin	Competing Elicitor	IC_{50} nM
^{125}I -Cryptogein	Cryptogein	139 ± 18
	Cinnamomin	660 ± 98
	Capsicein	195 ± 22
	Parasiticein	100 ± 27
^{125}I -Cinnamomin	Cryptogein	139 ± 18
	Cryptogein	240 ± 33
^{125}I -Capsicein	Cryptogein	251 ± 45
^{125}I -Parasiticein	Cryptogein	220 ± 20

AOS Production

AOS production was measured in response to increasing concentrations of elicitors (5–500 nM). The four elicitors induced AOS production in tobacco cell suspensions. Nevertheless, the kinetics and the intensity of this production were specific for each elicitor and depended on their concentration (Fig. 6). For example, 100 nM cryptogein induced a rapid, strong burst of AOS after a 10-min treatment (about $1200 \text{ nmol H}_2\text{O}_2 \text{ g}^{-1}$ fresh weight cells), and then the production of H_2O_2 decreased slowly. When the other elicitors were used at 100 nM, the AOS production reached a similar maximal value, which was delayed (30–40 min) and did not decrease significantly in the following 1 h. When lower concentrations of elicitors were used (10 nM) the production of AOS induced by cryptogein reached almost the same maximum value ($1000 \text{ nmol H}_2\text{O}_2 \text{ g}^{-1}$ fresh weight cells) after 10 min (Fig. 6). The oxidative burst induced by 10 nM cinnamomin appeared later (after 20 min) and had a lower intensity, whereas no significant burst was measured with 10 nM capsicein and parasiticein.

As previously observed with regard to the extracellular medium alkalization or the Ca^{2+} influx, cryptogein was the most efficient elicitor: the highest AOS production was achieved with 25 nM cryptogein, whereas 50 nM cinnamomin or greater than 500 nM acidic elicitors were necessary to obtain a maximal AOS production. AOS production was also measured to verify whether competition between acidic and basic elicitors led to a decrease of the effects of the most efficient basic elicitor. In vivo competition experiments on tobacco cells were performed using 25 nM cryptogein and various concentrations of capsicein (Fig. 7). AOS production due to the cryptogein treatment in the competition assay was scored by subtracting the AOS production contributed by capsicein alone from the total AOS production, at the concentration corresponding to that of the competition experiment. Cryptogein-induced AOS production was decreased by 40% when tobacco cells were simultaneously treated with 50 nM capsicein. With higher capsicein concentrations (100 and 250 nM) the AOS production induced by cryptogein was reduced by about 80% to 90%, showing effective in vivo competition.

Table III. Inhibition of ^{125}I -cryptogein-specific binding by elicitins after derivatization with nonradioactive iodine calculated from competition curves

Data are from Figure 3 and represent the averages \pm SD of three experiments.

	IC_{50}			
	I-Cryptogein	I-Cinnamomin	I-Capsicein	I-Parasiticein
^{125}I -Cryptogein	14.4 \pm 6.2	4.8 \pm 2.9	<i>nm</i>	20.1 \pm 3.8

DISCUSSION

In this study we compared the binding properties of four different elicitins on tobacco plasma membrane preparations and their effects on tobacco cells. The four elicitins produced by *Phytophthora* have a high degree of sequence conservation (74%) and should therefore have a similar structure (Bouaziz et al., 1994; Boissy et al., 1996). Nevertheless, they are classified into acidic and basic elicitins based on their pI. An elicitin necrotic index was established that correlated with pI, and basic elicitins had high necrotic activity (Pernollet et al., 1993). The different level of activities of elicitins should not depend on their stability: elicitin uptake experiments in tobacco with labeled cryptogein demonstrated high elicitin stability in vivo, because the protein could be recovered in a nondegraded form 8 d after the onset of treatment (Keller et al., 1996). Furthermore, the in vitro stability of elicitins is a general property of these proteins (resistance to boiling and to trypsin treatment; data not shown), probably because of their similar structure.

Using two basic elicitins (cryptogein and cinnamomin) and two acidic elicitins (capsicein and parasiticein), we examined several aspects of their structure-function relationship. We first looked at binding-affinity constants and found that all four ^{125}I -labeled elicitins had a similar K_d , ranging from 5.8 to 13.5 nM, and that the number of binding sites on the plasma membrane was between 234 and 403 fmol/mg plasma membrane proteins (Table I). These binding constants were very close to those previously determined by Wendehenne et al. (1995) using tobacco plasma membrane preparations and ^{125}I -labeled cryptogein: a K_d of 2 nM and 220 fmol/mg binding sites on plasma membrane proteins. Differences were expected because we used a different binding buffer suitable for cross-linking experiments. In the present study the data based on Scatchard plots suggest that elicitin-binding properties are equivalent. This assumption is further supported by experiments showing that the specific binding of each elicitin was optimal at pH 7.0, regardless of its acidic or basic nature (Fig. 2).

Competition experiments were performed to verify whether elicitins bound to the same high-affinity binding sites. Results demonstrated that each nonradioactive iodinated elicitin was able to compete with ^{125}I -cryptogein, and the corresponding IC_{50} values were similar (between 4.8 and 20.1 nM) and close to the K_d of cryptogein (Table III). However, an apparent better affinity of iodinated versus noniodinated elicitins was suggested by the comparison of the IC_{50} values, which were higher for unlabeled iodinated elicitins (Tables II and III). Consequently, the affinity of

noniodinated elicitins for binding sites might be lower than those determined for iodinated elicitins. At this stage we are unable to explain why iodination increases affinity and why this increase in affinity is not linked to an increase in activity. Elicitins possess four to five Tyr residues with various degrees of accessibility (based on the cryptogein structure; Boissy et al., 1996). Only two conserved Tyr residues (Tyr-47 and Tyr-87) are partly accessible to the solvent, and only one is fully accessible in basic elicitins (Tyr-12). The iodination of one of these residues could modify the binding properties if the residue lies within or in close vicinity to the interacting domain.

The fact that elicitins showed similar K_d constants and IC_{50} values regardless of the treatment (iodination or absence of iodination) demonstrated that they were able to interact with the same binding sites on plasma membranes. Binding experiments have also indicated that nonconserved, charged amino acids that confer acidic or basic pIs were probably not involved in binding. Therefore, binding relies on invariant amino acid interactions, which is in agreement with results of radiographic crystallography of cryptogein indicating that elicitins may share a very similar tertiary structure and a beak-like motif resulting from the arrangement of an Ω -loop and a β -sheet essentially composed of invariant residues. This conserved structure could be a major recognition site for a putative receptor (Boissy et al., 1996).

Elicitors of different chemical origin have been reported to interact with high affinity with plasma membrane com-

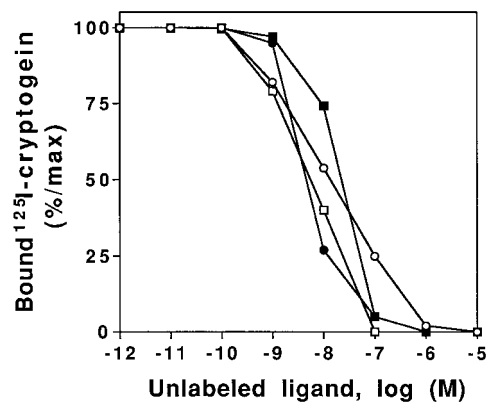


Figure 3. Inhibition of ^{125}I -cryptogein binding to the plasma membrane by increasing concentrations of various elicitins (competitor). Fifty micrograms of plasma membrane proteins was incubated with 10 nM ^{125}I -cryptogein and increasing concentrations of cryptogein (\circ), cinnamomin (\bullet), capsicein (\square), or parasiticein (\blacksquare). Data are expressed as percentages of the specific binding of ^{125}I -cryptogein, each point being an average of triplicate measurements.

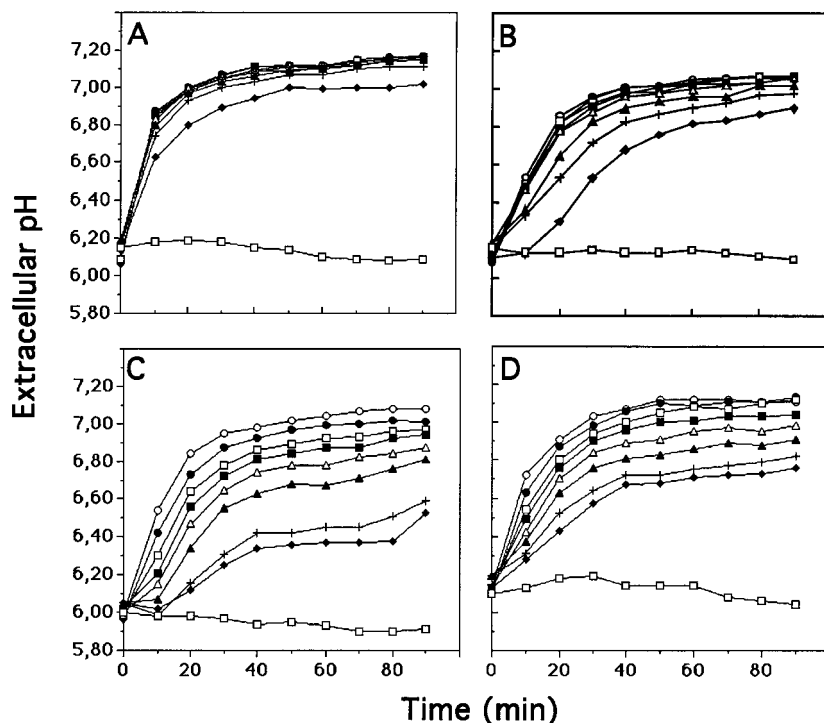


Figure 4. Effects of increasing concentrations of elicitors on extracellular pH of tobacco cell suspensions. Cells were treated with cryptogein (A), cinnamomin (B), capsicein (C), or parasiteicin (D). Elicitor concentrations were 5 nM (◆), 10 nM (+), 25 nM (▲), 50 nM (△), 75 nM (■), 100 nM (□), 250 nM (●), and 500 nM (○). Experiments were repeated three times. The figure corresponds to a representative experiment.

ponents. Chemically pure carbohydrates and glycopeptides or proteinaceous elicitors bound with K_d values at or below the nanomolar range (Cheong and Hahn, 1991; Cosio et al., 1992; Basse et al., 1993; Shibuya et al., 1993; Baureithel et al., 1994; Nürnberger et al., 1994; Kooman-Gersmann et al., 1996). Evidence for high-affinity binding sites is usually correlated with biological activities, confirming a linkage with a cellular signal transduction chain. A strong relationship between competitor abilities of various elicitors and plant defense responses has been established previously (Cheong and Hahn, 1991; Basse et al., 1993; Baureithel et al., 1994; Nürnberger et al., 1994).

A positive binding/activity correlation has also been reported using β -glucan elicitors and six species of the same plant family (Cosio et al., 1996). However, since elicitor binding seems to be a prerequisite for the induction of the plant-defense response, this interaction is not sufficient for elicitor-induced plant-defense mechanisms. The race-specific AVR9 peptide elicitor from *Cladosporium fulvum* bound on plasma membrane preparations from both fungus-resistant and fungus-susceptible tomato cultivars and from other solanaceous plants (Kooman-Gersmann et al., 1996). It was suggested that in the susceptible cultivar binding properties were related to the presence of homologs of the resistance gene *Cf-9*, which characterized the resistant tomato cultivar. In tomato cells the same binding site was shown to bind a glycopeptide (elicitor), inducing ethylene production and the *N*-linked glycans released from this glycopeptide (Basse et al., 1993). The *N*-linked glycans act as suppressors of ethylene production.

A comparison of the previously described effects of elicitors on whole tobacco plants (see introduction) and of the elicitor-binding properties did not indicate a strict relation-

ship between elicitor perception and plant response. Therefore, we looked at early physiological events in tobacco cells following elicitor perception, which would reflect a modulated signal transduction step dependent on an elicitor complex leading to different plant defense levels. We chose to measure the effects of the four elicitors on Ca^{2+} influx (which is, to our knowledge, the earliest event induced by cryptogein in tobacco cells after protein phosphorylation; Tavernier et al., 1995b) and on the subsequent Ca^{2+} -dependent events extracellular medium alkalization and AOS production.

Using $^{45}\text{Ca}^{2+}$ as a tracer we observed that the four elicitors were able to induce Ca^{2+} influx. Nevertheless, cryptogein triggered a much more intense Ca^{2+} influx compared with the other elicitors (Fig. 5). The ability of elicitors to trigger different levels of Ca^{2+} influx was confirmed using pH conditions (buffered medium, pH 7.0) that allowed similar binding with the four elicitors (Fig. 5C). The intensity of the Ca^{2+} influx was not associated with the basic or acidic characters of elicitors, because the basic cinnamomin has an effect on Ca^{2+} influx similar to those of acidic elicitors.

Extracellular pH changes were also monitored in response to the four elicitors. The comparison of the effects of either 10 or 100 nM elicitor indicated that cryptogein was always the most efficient. At low concentrations all elicitors except cryptogein showed a lag phase of at least 10 min before the pH of the extracellular medium increased (Fig. 4). At higher concentrations the lag phase was shortened and the maximal pH variation was reached after 60 min of treatment with the four elicitors, although with different initial rates. The lowest concentrations necessary to achieve maximal pH increase with the highest rates were 10 and 50

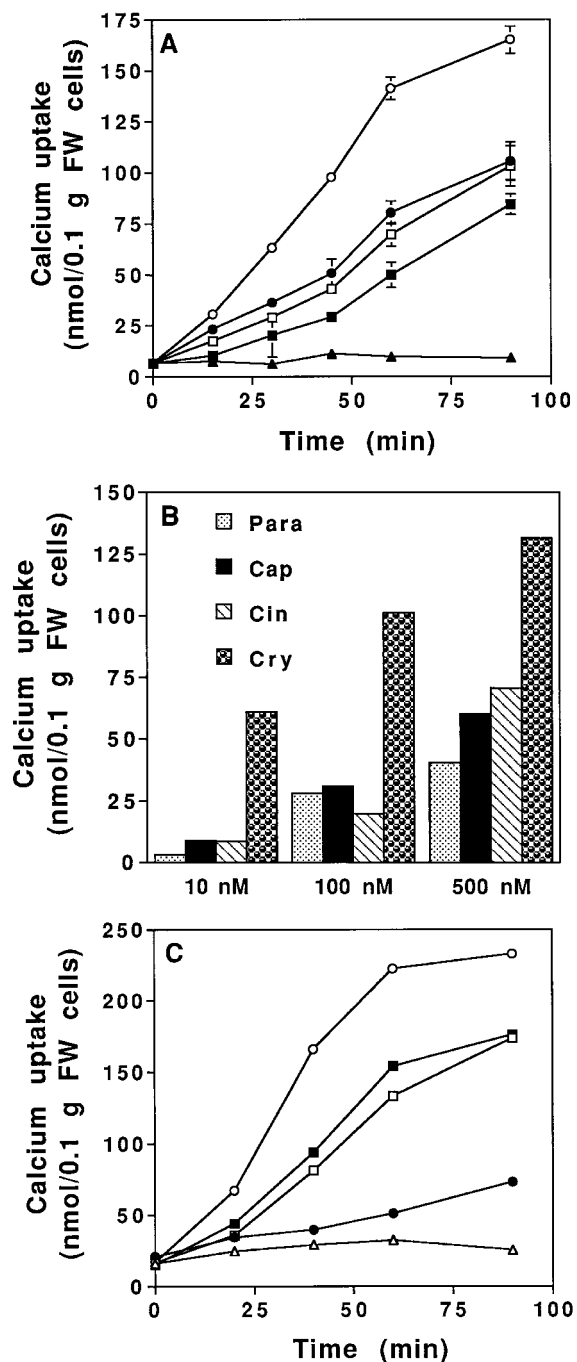


Figure 5. Effect of various concentrations of elicitors on $^{45}\text{Ca}^{2+}$ influx into tobacco cells. A, Time course of $^{45}\text{Ca}^{2+}$ uptake by tobacco cells after treatment with 500 nM elicitors: cryptogein (○), cinnamomin (●), capsicein (□), and parasiticein (■); control cells (▲) in a 2 mM HEPES low-buffered medium, pH 5.75. B, Effects of increasing concentrations of elicitors on $^{45}\text{Ca}^{2+}$ uptake into tobacco cells after 1 h of incubation with cryptogein (Cry), cinnamomin (Cin), capsicein (Cap), or parasiticein (Para) in a 2 mM HEPES low-buffered medium, pH 5.75. C, Time course of $^{45}\text{Ca}^{2+}$ uptake by tobacco cells after treatment with 100 nM elicitors: cryptogein (○), cinnamomin (●), capsicein (□), or parasiticein (■); control cells (▲) in a 50 mM HEPES medium buffered at pH 7.0. Each point corresponds to a duplicate assay. Results are means \pm SD. Specific activity of $^{45}\text{Ca}^{2+}$ = 6.6 GBq/mol. FW, Fresh weight.

nM for cryptogein and cinnamomin, respectively, and about 500 nM for capsicein and parasiticein. Basic elicitors appeared at least 10 times more efficient than acidic elicitors in inducing extracellular pH changes. The same conclusions were drawn when measuring the AOS production induced by the four elicitors (Fig. 6). The concentrations necessary to induce maximal AOS production were similar to those necessary to induce the maximal extracellular pH increase (25 and 50 nM for cryptogein and cinnamomin, respectively, and about 500 nM for the two acidic elicitors). The AOS burst increased concurrently with extracellular pH and was delayed for cinnamomin, capsicein, and parasiticein compared with cryptogein. However, at a high elicitor concentration (100 nM) AOS production reached the same level (approximately 1200 nmol H_2O_2 g^{-1} fresh weight cells) with the four elicitors, although with different kinetics.

Because the four elicitors bind to the same sites with the same affinity, one would expect that when mixed together an elicitor with low efficiency (acidic) would decrease the effects of a highly efficient (basic) elicitor. These competition assays required 20 nM basic elicitor and 10 to 50 times more acidic elicitor (200 nM to 1 μM). The acidic elicitor contributions at these concentrations were high and differences were particularly slight for extracellular pH measurements. Therefore, *in vivo* competition experiments were performed by measuring only the AOS production induced by cryptogein in competition with capsicein. Results showed that capsicein was also a competitor *in vivo*: 100 nM capsicein induced a 90% inhibition of the 25 nM cryptogein-induced AOS production, which supports an *in vivo* competition process.

Even when elicitors were tested on tobacco cell suspensions, differences in the intensity of the early effects of elicitor arose and could reflect the elicitor properties that were correlated with the electric charge of the elicitors. One explanation for the different biological activities of elicitor would be that the whole elicitor charge might restrict the acidic elicitor diffusion at the 5.8 physiological pH in the negatively charged cell wall, resulting in lower amounts of acidic elicitors interacting with binding sites. Alternatively, following binding between the elicitor conserved domain and the receptor, the amino acid electric charges that characterized the unconserved domain could trigger different conformational changes in the receptor, leading to variations in the intensities and kinetics of responses at the cellular level.

Conversely, the formation of various oligomeric forms of the receptor could be induced by elicitors, as has been reported for animal polypeptide hormones, cytokines, and growth factors (Heldin, 1995). For example, the different isoforms of platelet-derived growth factor induced different dimeric forms of receptors (homodimeric and heterodimeric forms), resulting in not only modulated response intensities but also additional signal transduction molecule interactions. Such mechanisms could also explain the behavior of the different elicitors at the cellular level. In addition to the beak-like motif, elicitors contain two other regions around residues 13 and 87 whose charge distribution could be correlated with the level of biological activity

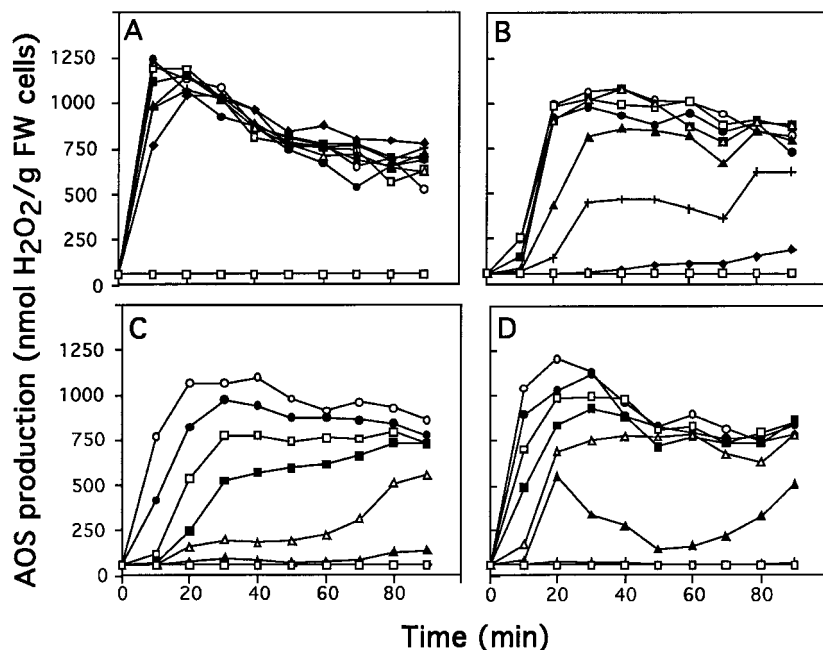


Figure 6. AOS production in tobacco cell suspensions ($\text{nmol H}_2\text{O}_2 \text{ g}^{-1}$ fresh weight cells) treated with increasing concentrations of elicitors: 5 nM (\blacklozenge), 10 nM ($+$), 25 nM (\blacktriangle), 50 nM (\triangle), 75 nM (\blacksquare), 100 nM (\square), 250 nM (\bullet), and 500 nM (\circ). Cells were treated with cryptogein (A), cinnamomin (B), capsicein (C), or parasiticein (D). The experiment was repeated three times. The figure corresponds to a representative experiment. FW, Fresh weight.

of elicitin (Boissy et al., 1996). These regions could modulate the intensity of tobacco cell responses after binding on the receptor. Presently, experiments are in progress to identify the receptor of elicitins. After this receptor has been identified, the interactions between ligand and receptor will be studied at the molecular level.

In conclusion, fine regulation of signal transduction is a topic of major interest, although many aspects remain obscure because of a lack of information about the many processes involved. Only recently was a β -glucan elicitor-

binding site characterized at the molecular level (Umemoto et al., 1997). An integrated view of the transduction pathway includes the analysis of the environment of the receptor and knowledge of the molecules recruited to transduce signals such as protein kinases, protein phosphatases, or specific ion channels.

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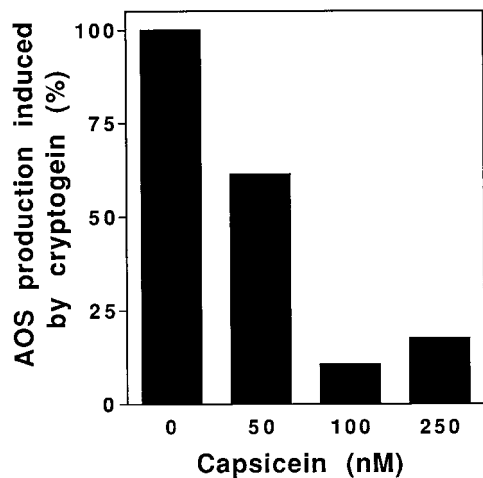


Figure 7. In vivo competition experiment using 25 nM cryptogein and increasing capsicein concentrations. The AOS production induced by 25 nM cryptogein was referred to as 100%. AOS production due to cryptogein in competition experiments was deduced by subtracting from the total AOS production the AOS contribution of capsicein alone used at the concentration corresponding to that used in the competition experiment. The AOS production was measured after treatment for 15 min with elicitors.

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