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# Elicitor-Induced Changes in $Ca^{2+}$ Influx, $K^+$ Efflux, and 4-Hydroxybenzoic Acid Synthesis in Protoplasts of *Daucus carota* L.[1]

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**Suspension-cultured carrot cells (*Daucus carota*) and their protoplasts respond to a fungal elicitor prepared from the culture medium of *Pythium aphanidermatum* by accumulating 4-hydroxybenzoic acid (4-HBA). Protoplasts release the compound into the culture medium. Using $^{45}CaCl_2$ as a tracer, we were able to demonstrate that the secretion of 4-HBA is preceded by a rapid increase in the $Ca^{2+}$ influx and a concomitant $K^+$ efflux. If the increased $Ca^{2+}$ influx was prevented by ethyleneglycol-bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 4-HBA synthesis was inhibited by 70%. These results are discussed with regard to signal transduction from the plasma membrane to the nucleus of carrot protoplasts.**

In higher plants there is growing evidence that changes in cytosolic free $Ca^{2+}$ play an important role in a number of different physiological processes induced by external stimuli such as light, growth regulators, gravity, and elicitors. Cytosolic $Ca^{2+}$ is considered to be a second messenger during signal transduction from the cell surface to internal compartments of the plant cell.

Fungal elicitors trigger biosynthetic pathways leading to the de novo synthesis of phytoalexins. In cell cultures of carrot (*Daucus carota*) (Kurosaki et al., 1987) and soybean (Stäb and Ebel, 1987), the $Ca^{2+}$ ionophore A23187 can mimic the elicitor-induced synthesis of phytoalexins. Moreover, results of experiments with the $Ca^{2+}$ channel blocker nifedipine (Waldmann et al., 1988) suggest that the influx of $Ca^{2+}$ is a critical event in elicitor-induced signal transduction. Measurements of ion concentrations in the culture medium of suspension-cultured cells using ion-selective electrodes showed an elicitor-induced decrease in $Ca^{2+}$ accompanied by an increase in $K^+$ ions paralleled by an alkalization of the medium (Colling, 1991; Conrath et al., 1991). These ion fluxes across the plasma membrane are often followed by a depolarization of the plasma membrane (Pellissier et al., 1986), lipid peroxidation (Peever and Higgins, 1989), and an "oxidative burst" that may involve $H_2O_2$ production upon elicitor treatment (Apostol et al., 1989).

In plant cells, it is difficult to discriminate between the actual ion fluxes across the plasma membrane and the ion exchange capacity of the wall polysaccharides (Hepler and Wayne, 1985; Kauss, 1987). Therefore, a wall-less system expressing phytoalexin biosynthesis upon elicitor treatment is desirable for ion flux measurements. In a previous report, we described a protoplast system from carrot that is inducible by a fungal elicitor from *Pythium aphanidermatum* (Schnitzler and Seitz, 1989). Treatment with the fungal elicitor resulted in the stimulation of the phenylpropanoid pathway, as was shown by the de novo synthesis of Phe ammonia-lyase followed by the secretion of 4-HBA into the culture medium. Concomitantly with the regeneration of a new cell wall, 4-HBA is covalently linked to wall polysaccharides as is the case in intact suspension-cultured cells. In the present communication, we demonstrate that the synthesis and secretion of 4-HBA by carrot protoplasts are accompanied by rapid increases in the influx of $^{45}Ca^{2+}$ and an efflux of $K^+$ ions a few minutes after addition of the elicitor.

## MATERIALS AND METHODS

### Chemicals and Radiochemicals

$^{45}CaCl_2$ (0.68 GBq $mg^{-1}$) was from Amersham Buchler (Braunschweig, Germany). Catalase was purchased from Boehringer (Mannheim, Germany).

### Cell Cultures

The experiments were carried out with an anthocyanin-containing cell line of *Daucus carota* L. Cell cultures were cultivated as previously described (Noé et al., 1980).

### Isolation of Protoplasts

The protoplasts were isolated by slightly modifying a procedure previously described (Schnitzler and Seitz, 1989). Cells (5 g of fresh weight) were incubated in 50 mL of buffered PC-6 medium at pH 5.6 containing 1% (w/v) cellulase (Onozuka RS; Yakult Honsha Co. Ltd., Tokyo, Japan), 0.5% (w/v) pectinase (Serva, Heidelberg, Germany), and 0.2% (w/v) BSA (Sigma, München, Germany) for 10 h at 26°C. All other steps were as described previously (Schnitzler and Seitz, 1989).

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Abbreviation: 4-HBA, 4-hydroxybenzoic acid.

### Cultivation of Protoplasts

Purified protoplasts were counted in a Fuchs-Rosenthal hematocytometer and checked for viability by staining with Evans blue (0.1%, w/v; Sigma) (Schnitzler and Seitz, 1989). The viability of protoplasts after 48 h was determined in all experiments described in this paper.

To measure the accumulation of 4-HBA, the suspension was brought to a cell titer of $0.5 \times 10^6$ protoplasts $mL^{-1}$. The samples were cultivated in portions of 12 mL in Petri dishes at 26°C in the dark. The influx measurements with $^{45}Ca^{2+}$ and the $K^+$ determinations were carried out with 2-mL suspensions adjusted to a cell titer of $2.4 \times 10^6$ protoplasts $mL^{-1}$.

### Elicitor Preparation

*Pythium aphanidermatum* was cultured as described by Schnitzler and Seitz (1989). The elicitor was prepared by concentrating 2 L of culture medium to 10 mL as previously described (Schnitzler et al., 1992). This preparation is referred to in this report as fungal elicitor. It contained 1.9 mg $mL^{-1}$ of carbohydrates (Glc equivalents) and 0.37 mg $mL^{-1}$ of protein.

### $^{45}Ca^{2+}$ Influx Measurements

The PC-6 medium used for protoplast isolation and cultivation contains 6 mM $CaCl_2$. During the first 10 min after addition of labeled $Ca^{2+}$, even in the absence of the elicitor, an apparent increase in $Ca^{2+}$ influx occurred (data not shown), which might have been due to an exchange of labeled and unlabeled $Ca^{2+}$ in the protoplasts and/or their respective plasma membranes. Similar results were obtained with protoplasts of *Amaranthus* (Rengel and Elliot, 1992). Therefore, 2 mL of protoplast suspension ($2.4 \times 10^6$ protoplasts $mL^{-1}$ of PC-6 medium) were preincubated for 10 min with $^{45}CaCl_2$ (0.68 GBq $mg^{-1}$ adjusted to a final concentration of $8 \times 10^5$ dpm $mL^{-1}$) before the addition of the elicitor (controls with equal volumes of PC-6 medium instead of the elicitor) to obtain an equilibrium of labeled and unlabeled $Ca^{2+}$ (see above). To prevent sedimentation of protoplasts, the suspension was gently agitated. After various incubation periods in the presence of the elicitor, duplicate samples of 120 μL were withdrawn and diluted with the stop solution (40 mM Hepes [pH 7.6] containing 0.7 M NaCl, 16 mM $MgCl_2$, and 12 mM EGTA) to a final volume of 160 μL (Hopp and Seitz, 1987). Aliquots (100 μL) were layered on 150 μL of silicon oil (AP150/AR200, 1:1, v/v; Drawin Vertriebs GmbH, Ottobrunn, Germany) and centrifuged as previously described (Hopp and Seitz, 1987) to separate protoplasts from the aqueous incubation medium. After the vials were frozen in liquid nitrogen, the bottom of the reaction vial (400 μL, 5 × 40 mm) was chopped off with a razor blade and counted in 5 mL of Lumagel (Baker Chemicals, Deventer, The Netherlands) in a liquid scintillation counter.

### Determination of $K^+$ by Atomic Absorption Spectroscopy

All experiments were performed as described in the preceding section with the exception that the stop solution contained 4 mM EDTA instead of 12 mM EGTA. The bottom of the reaction vial with the protoplast pellet was placed in 20 mL of double-distilled water to lyse the protoplasts. After centrifugation at 6000*g* for 10 min, 18 mL of the supernatant were mixed with 2 mL of a CsCl solution (10 g $mL^{-1}$) and used for $K^+$ determination in an atomic absorption spectrometer (model 1272; Beckman Instruments, München, Germany) (Waldmann et al., 1988). The $K^+$ concentrations were calculated using $KNO_3$ as a standard. For the transient time course (see Fig. 2b), the samples for $^{45}Ca^{2+}$ determinations were taken at 50 min, when the influx of $Ca^{2+}$ decreased, whereas the cellular $K^+$ concentration was measured most effectively using atomic absorption spectroscopy after 80 min.

### Determination of 4-HBA

The 4-HBA content of the protoplasts and of the culture media was determined as previously described (Schnitzler and Seitz, 1989) with slight modifications: the butanol extract from the culture medium was dried in a stream of filtered air, and the resulting residue was redissolved in methanol:water (1:1, v/v) and analyzed by HPLC according to the method of Gleitz et al. (1991).

The 4-HBA accumulation was always determined at its maximum. In experiments concerning the minimum time requirements for stable expression of the elicitor effect, 4-HBA was measured 36 h after elicitation. In all other experiments, it was determined after 48 h.

### $H_2O_2$ Determination and Catalase Treatment

$H_2O_2$ in the medium of suspension-cultured cells and protoplasts was measured by chemiluminescence of luminol as described by Schwacke and Hager (1992). Catalase (100 units $mL^{-1}$ of protoplast suspension) and the elicitor were added simultaneously to the protoplast suspension.

### Analytical Methods

The carbohydrate concentrations of elicitor preparations were determined by the phenol-sulfuric acid method (Dubois et al., 1956) using Glc as a standard. Protein was determined according to the method of Bradford (1976) using BSA as a standard. All experiments were replicated at least two times, and all data presented are means of at least duplicates.

## RESULTS

### Minimum Time Requirement for Stable Expression of the Elicitor Effect

Carrot protoplasts respond to treatment with a fungal elicitor by releasing 4-HBA into the culture medium. This effect reaches a maximum 36 to 48 h after onset of elicitation. To determine the minimum period of exposure necessary to elicit a stable expression of the 4-HBA pathway, the fungal elicitor was removed after different times from the culture fluid by changing the medium three times.

As can be seen in Figure 1, at least 10 min of exposure are sufficient to induce a marked increase in the secretion of 4-HBA into the medium. This effect is maximal after 40 min of exposure to the elicitor; it does not change upon further treatment.

### $Ca^{2+}$ Fluxes after Elicitor Treatment

To show whether the 4-HBA secretion is associated with changes in the $Ca^{2+}$ fluxes across the plasma membrane, flux measurements were initiated using $^{45}CaCl_2$. After preincubation for 10 min (see "Materials and Methods"), the radioactivity remained constant for 100 min (Fig. 2a). Only in some experiments did the baseline ascend slightly. This basal activity was 25 to 60 nmol $10^{-6}$ protoplasts. However, in elicited protoplasts, the $Ca^{2+}$ influx started to increase 3 min after onset of elicitor treatment (data not shown). In most of the experiments, the uptake of $^{45}Ca^{2+}$ increased continuously to a level 2 or 3 times higher than that in nontreated control protoplasts (Figs. 2a and 3a). In some experiments (Fig. 2b), a transient influx is observed with a maximum between 30 and 50 min after onset of elicitation. Based on the measured influx of labeled $Ca^{2+}$, an influx of 50 to 70 nmol of $Ca^{2+}$ has been calculated. The average diameter of protoplasts has been determined by microscopic measurements to be 26 $\mu$m. This value was used to calculate the average volume of the protoplasts and the concentration changes, which were 3 to 6 mM $h^{-1}$.

### $K^+$ Release after Elicitor Treatment

After incubation of the protoplasts with the elicitor, $Ca^{2+}$ was taken up, and at the same time, the intracellular $K^+$ concentration decreased during the incubation period of 150 min with the elicitor (Fig. 3b). The cellular $K^+$ concentration of control protoplasts did not change significantly during the entire period. In Figure 3a, the corresponding $Ca^{2+}$ influx is shown. Based on the same calculation as for $Ca^{2+}$ (see above), the $K^+$ concentration in the total cell was about 250 mM and changed by about 70 mM $h^{-1}$. These values correspond nicely to literature data (Hepler and Wayne, 1985). Thus, the $K^+$ efflux across the plasma membrane was 10 to 20 times higher than the $Ca^{2+}$ influx.

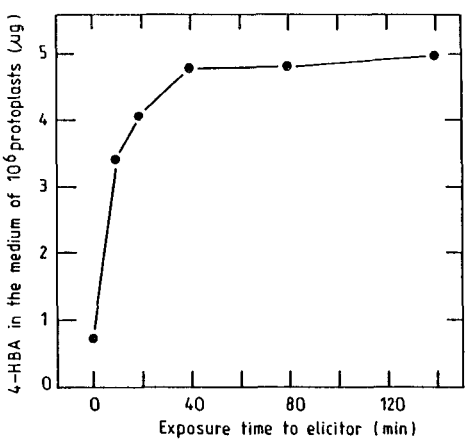


**Figure 1.** Influence of the period of exposure to an elicitor from *P. aphanidermatum* on 4-HBA synthesis by protoplasts. Samples (10 mL) of a protoplast suspension were treated with an elicitor from the concentrated culture medium (20 $\mu$L $10^{-6}$ protoplasts) of the fungus. At the times indicated, the elicitor was removed by washing. After 36 h, the amount of 4-HBA was determined by HPLC.

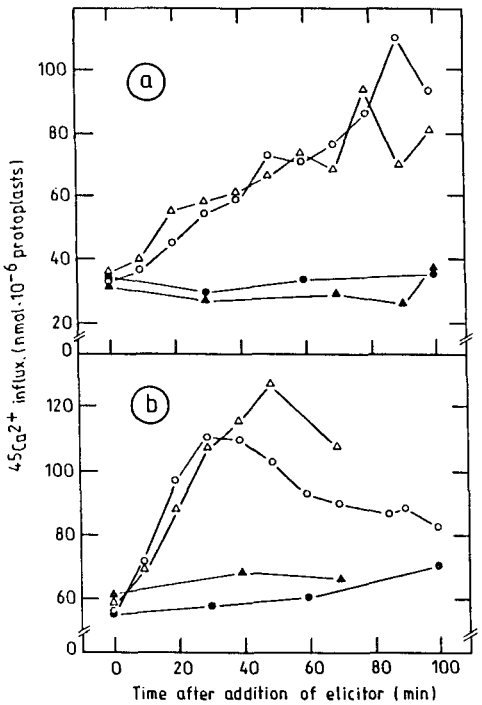


**Figure 2.** Time course of $^{45}Ca^{2+}$ influx by protoplasts after treatment with an elicitor. The protoplast suspension was preincubated for 10 min in the presence of the radiotracer and then 40 $\mu$L of the elicitor per $10^6$ protoplasts were added. The control received the same volume of medium instead of the elicitor. Every 10 min, aliquots were withdrawn and analyzed for $^{45}Ca^{2+}$ in the protoplasts by means of the silicon oil technique and liquid scintillation counting. Four independent experiments are shown. Each point is the mean value of duplicates. The maximum variation was 15 nmol. Of 15 experiments, 2 showed the transient temporal pattern depicted in b.

### Elicitor Concentration Dependency of $Ca^{2+}/K^+$ Fluxes and Secretion of 4-HBA

As shown in Figure 4, the ion fluxes as well as the secretion of 4-HBA are affected by the fungal elicitor in a concentration-dependent manner. Increasing amounts of the elicitor (up to 10 $\mu$L of elicitor extract $10^{-6}$ protoplasts) triggered a linear increase in the amount of 4-HBA secreted into the culture medium, as determined 48 h after onset of elicitation (Fig. 4a). At higher elicitor concentrations (20–80 $\mu$L of the medium extract $10^{-6}$ protoplasts), the 4-HBA concentration in the protoplast medium reached a maximum of 20 $\mu$g $10^{-6}$ protoplasts. The ion fluxes of $^{45}Ca^{2+}$ and $K^+$ were also dependent on the elicitor concentration in the incubation medium (Fig. 4b). During the period of observation, no saturation in the $K^+$ flux was observed, but the $Ca^{2+}$ flux showed a slight tendency to saturation.

As shown in Table I, the viability measured by Evans blue staining did not change upon treatment with the fungal elicitor.

### Influence of EGTA on the Elicitor-Induced $Ca^{2+}$ Influx and the Accumulation of 4-HBA

To demonstrate the possible role of $Ca^{2+}$ in transducing the elicitor signal for 4-HBA secretion, experiments with the

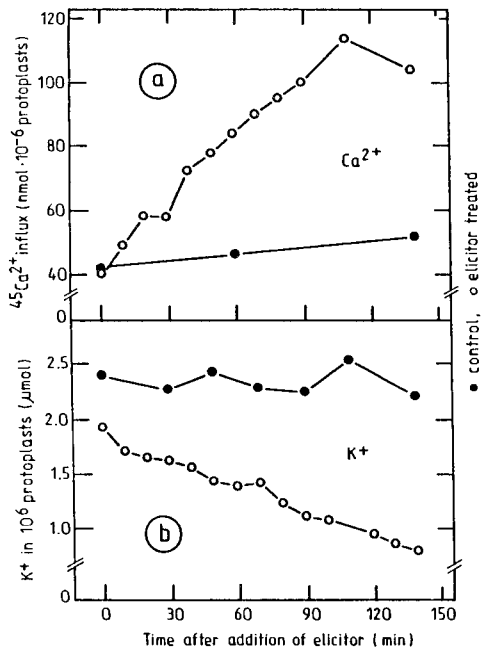


**Figure 3.** Changes in the intracellular $K^+$ concentration of protoplasts after elicitor treatment compared to $^{45}Ca^{2+}$ influx. The protoplasts were collected at the times indicated and separated from the aqueous medium with the silicon oil technique (see "Materials and Methods"). The pellet was treated with double-distilled water, and the resulting solution was used for $K^+$ determination by means of atomic absorption spectroscopy in the presence of CsCl using $KNO_3$ as a standard (b). The $Ca^{2+}$ influx (a) was determined as described in the legend to Figure 2. Representative results from four independent experiments are shown. Each point is the mean value of duplicates (variation in $K^+$ fluxes was between 0.05 and 0.4 $\mu$mol $10^{-6}$ protoplasts).

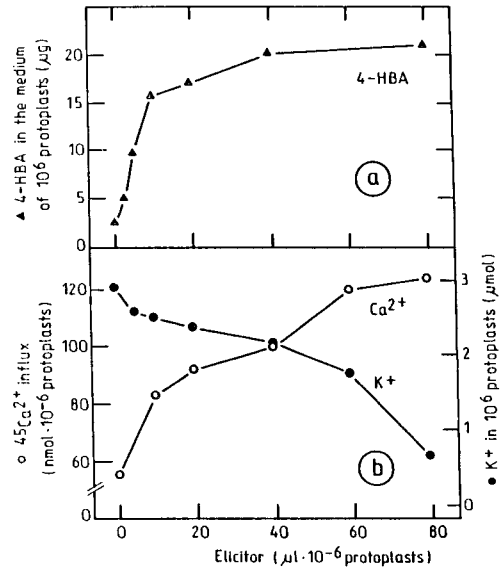


**Figure 4.** Comparison of 4-HBA synthesis (a) and $^{45}Ca^{2+}$ influx and $K^+$ release (b) by protoplasts as a function of the elicitor concentration in the medium. The amount of 4-HBA was determined 48 h after the addition of the elicitor. The $^{45}Ca^{2+}$ influx was measured 50 min after elicitor treatment. The intracellular $K^+$ concentration was determined 80 min after elicitation. Each point is the mean value of duplicates (maximum variation 10%).

preferential $Ca^{2+}$ chelator EGTA were performed. The results of this series of experiments are depicted in Figure 5. When the amount of exchangeable $Ca^{2+}$ in the culture medium was decreased with 7 mM EGTA, the elicitor-induced influx of the ion was inhibited within a few minutes, and $Ca^{2+}$ was released to the level of control protoplasts. The viability of protoplasts was monitored during a cultivation period of 48 h by Evans blue staining. At the concentrations indicated and within the period of observation, neither elicitor treatment nor complexing of $Ca^{2+}$ significantly influenced the viability of the protoplasts. It was 83% after 48 h. The removal of extracellular $Ca^{2+}$ from the medium and the inhibition of the $Ca^{2+}$ influx also inhibited the secretion of the 4-HBA by 70% (Fig. 5, right). Even in experiments with higher concentrations of EGTA (10.5 mM), a remainder of about 30% of 4-HBA was always observed (data not shown). In the control in the presence of 7 mM EGTA, viability and 4-HBA secretion of protoplasts remained unchanged.

### $H_2O_2$ Production after Elicitation

An alternative type of plant cell response to infection or elicitor treatment includes the secretion of $H_2O_2$ into the medium, as has been described for various systems (Doke, 1985; Apostol et al., 1989; Schwacke and Hager, 1992). Our carrot cells did not respond to elicitor treatment by $H_2O_2$ production, and catalase treatment of the protoplasts did not inhibit $^{45}Ca^{2+}$ influx or 4-HBA accumulation (data not shown).

## DISCUSSION

We have shown that carrot protoplasts respond to exposure of at least 10 min with a fungal elicitor from *P. aphanidermatum* by synthesizing and secreting 4-HBA into the culture medium. The 4-HBA was determined 48 h after addition of the elicitor. This metabolic event is preceded by an elicitor-dependent $Ca^{2+}$ influx and a concomitant efflux of $K^+$ ions. This net influx of $Ca^{2+}$ was shown by direct measurements using $^{45}CaCl_2$ as a tracer. The elicitor-induced $Ca^{2+}$ influx is

**Table I.** *Viability of protoplasts (%) after elicitor treatment determined by staining of nonviable protoplasts with Evans blue and counting in a Fuchs-Rosenthal hematocytometer*

Each value is the mean of duplicates.

| Time | Percentage of Viability | |
|---|---|---|
| | Control | Elicitor (25 µL $10^{-6}$ protoplasts) |
| *h* | | |
| 0 | 96.0 | 96.0 |
| 12 | 92.3 | 91.0 |
| 24 | 90.5 | 87.3 |
| 48 | 87.8 | 88.3 |

inhibited, and the 4-HBA synthesis is decreased to 30% of normal in the presence of EGTA.

There are some examples of measurements of $Ca^{2+}$ influx with suspension-cultured cells using $^{45}Ca^{2+}$ as a tracer. Atkinson et al. (1990) observed increased $Ca^{2+}$ uptake during the hypersensitive reaction of tobacco cells after bacterial infection. Conrath et al. (1991) and Colling (1991), using parsley, also showed increased $Ca^{2+}$ fluxes in the presence of an elicitor. In this type of experiment, wall-bound $Ca^{2+}$ complicates the interpretation of the data. Because of these problems, we established a protoplast system from carrot and measured the elicitor-induced $^{45}Ca^{2+}$ influx. In contrast to suspension-cultured cells, protoplasts very often do not retain their responsiveness to elicitors because of the effects of endogenous elicitors and the effects of the fungal hydrolases used for the preparation of protoplasts; it has been shown for *Ricinus* that the enzymic hydrolysis of the wall mimics a fungal infection and leads to an induction of casbene synthesis (Lee and West, 1983). As already described in a previous paper (Schnitzler and Seitz, 1989), carrot protoplasts show the same response to the elicitor from *P. aphanidermatum* as the respective suspension-cultured cells.

The regulatory role of $Ca^{2+}$ depends on transient changes in the cytosolic free $Ca^{2+}$; this has been referred to as "amplitude" modulation (Callaham and Hepler, 1991). In most of our experiments, the influx proceeds continuously, but in about 10% of the experiments (Fig. 2b), transient increases with a maximum at 30 to 50 min after application of the elicitor were observed. With regard to $Ca^{2+}$ as a second messenger, a transient temporal pattern could be expected. To maintain a low cytosolic $Ca^{2+}$ concentration, the cells may actively transport the ion to the vacuole, the ER, and the apoplast. A continuous influx observed in the majority of our kinetic studies does not, therefore, automatically imply a permanent increase in cytosolic free $Ca^{2+}$ (Sanders et al., 1990).

From our influx studies and the average volume of the carrot protoplasts (see "Materials and Methods"), the $Ca^{2+}$ concentration after 1 h has been calculated to be 4.9 mM. This value is in the same range (5.7 mM) as in guard cells of *Commelina communis* (MacRobbie, 1989). With a $Ca^{2+}$-specific fluorescent dye (Indo-1) in the same system, a cytosolic free $Ca^{2+}$ concentration of 100 to 600 nM was determined (Fricker et al., 1990). This resting level of about 500 nM was also found in other plant cells (Gilroy et al., 1986; Callaham and Hepler, 1991). The discrepancy between the above-mentioned changes and the total cytosolic free $Ca^{2+}$ concentration may be explained by intracellular $Ca^{2+}$ sequestration and to a certain degree by binding to the plasma membrane.

This issue can only be resolved by direct measurements of cytosolic free $Ca^{2+}$. However, the method used in the study reported here is not suitable for direct determinations of the cytosolic free $Ca^{2+}$. At present, we are unable to determine the cytosolic free $Ca^{2+}$ because all attempts failed to introduce $Ca^{2+}$-specific, fluorescent probes into our carrot protoplasts, as was the case in many other systems (Callaham and Hepler, 1991). Because of the rigid cell wall and the cell turgor, it is difficult to measure cytosolic free $Ca^{2+}$ concentrations in plants. There are only a few examples where $Ca^{2+}$-sensitive fluorescent dyes have been introduced into cells using microinjection or electroporation (Gilroy et al., 1986). The microelectrode technique is difficult to apply to small cells. It has been successfully used for algal cells (Brownlee and Wood, 1986).

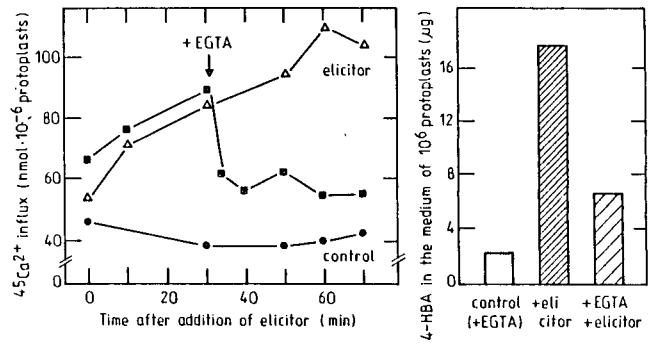


**Figure 5.** Left, Influence of EGTA on the elicitor-induced $^{45}Ca^{2+}$ influx. The protoplasts were treated with 7 mM EGTA 30 min after the onset of elicitor treatment (for influx measurements see Fig. 2). ●, Control in the absence of the elicitor and EGTA; ■, in the presence of the elicitor with the addition of 7 mM EGTA after 30 min; Δ, in the presence of the elicitor only (40 µL elicitor extract per $10^6$ protoplasts). Right, Comparison of the amount of 4-HBA in the culture medium of protoplasts after elicitor treatment (40 µL $10^{-6}$ protoplasts) and after simultaneous treatment with EGTA (7 mM) and the elicitor. The 4-HBA was determined by HPLC after 48 h. Control was in the presence of 7 mM EGTA (2.1 µg of 4-HBA $10^{-6}$ protoplasts). For a control in the absence of EGTA, see the value at 0 µL of the elicitor (2.5 µg of 4-HBA $10^{-6}$ protoplasts; Fig. 4a). Representative results from three independent experiments are shown. Each point is the mean value of duplicates (maximum variation 15%).

In addition to the $Ca^{2+}$ influx, elicitors may induce fluxes of other important ions. In suspension-cultured cells of different plant species, elicitation is followed by an increased $K^+$ export and an alkalization of the culture fluid (Waldmann et al., 1988; Colling, 1991; Conrath et al., 1991). During the hypersensitive reaction of tobacco cells after infection with *Pseudomonas syringae*, a $K^+$ export and an increased $Ca^{2+}$ uptake were also observed (Atkinson et al., 1990). After elicitor treatment of carrot protoplasts, the $K^+$ efflux is increased dramatically and inversely to the $Ca^{2+}$ fluxes. The amount of $K^+$ exported by the cell is much higher than the rate of $Ca^{2+}$ import. There are several reports of plant systems in which an increase in cytosolic $Ca^{2+}$ activates ion channels, especially $K^+$ channels (Hedrich and Schroeder, 1989; Schroeder and Hagiwara, 1989; Tester and MacRobbie, 1990).

The results presented in this communication are in agreement with the hypothesis that elicitor interaction with cells results, directly or indirectly, in ion channel opening. The connection of such ion flux changes with signal transduction and ultimate gene expression changes now needs to be explored.

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