Inhibition of Elicitor-Induced Phytoalexin Formation in Cotton and Soybean Cells by Citrate

IZYDOR APOSTOL, PHILIP S. LOW, PETER HEINSTEIN*, ROBERT D. STIPANOVIC, AND DAVID W. ALTMAN

Department of Chemistry (I.A., P.S.L.) and Department of Medicinal Chemistry and Pharmacognosy (P.H.), Purdue University, West Lafayette, Indiana 47907; and National Cotton Pathology Research Laboratory and Cotton Genetics and Grain Crops Research Unit, United States Department of Agriculture, Agricultural Research Service, P. O. Drawer JF, College Station, Texas 77841 (R.D.S., D.W.A.)

ABSTRACT

Addition of an elicitor preparation from Verticillium dahliae to soybean or cotton cell suspension cultures induces the formation of the phytoalexins, glyceollin or sesquiterpene aldehydes, respectively. Recent work (PS Low, PF Heinsteine 1986 Arch Biochem Biophys 249: 472-479) has shown that the induction of phytoalexin biosynthesis in these cells is preceded by rapid changes in the plant cell membrane which can be conveniently monitored by membrane associated fluorescent probes. Using this elicitation assay, we have found that citrate, a common metabolite of higher plants, acts as a potent inhibitor of elicitation when added prior to treatment with elicitor. The citrate concentration required to obtain a 50% inhibition of the elicitor-induced fluorescence transition in cultured cotton cells was found to be about 2 millimolar, while the concentration of citrate observed to inhibit elicitor-induced sesquiterpene aldehyde formation in the same cell suspensions was also 2 millimolar. Curiously, in the presence of elicitor, citrate at less than 1Dm concentrations increased cell mass accumulation significantly above control incubations without elicitor. A similar inhibition of glycine formation with an increase in cell mass accumulation was also observed upon addition of 1 to 5 millimolar citrate to soybean cell suspension cultures. The physiological significance of the inhibition by citrate of phytoalexin formation in plant cell suspensions was supported by the observation that a similar inhibition of sesquiterpene aldehyde formation occurs in cotton plantlets elicited by cold shock or V. dahliae stress. The specificity of citrate as an inhibitor of phytoalexin formation was demonstrated by data showing that other di- and tricarboxylic-acids did not inhibit, with the exception of malate which inhibited phytoalexin formation in soybean cells with roughly half the potency of citrate. These experiments not only demonstrate that citrate can act as a specific inhibitor of elicitation, but they further confirm the validity of monitoring elicitation and its modulation with fluorescent probes.

To prevent successful invasion by phytopathogenic fungi, plants have developed elaborate defense mechanisms which include the formation of potentially toxic secondary metabolites called phytoalexins (1, 3, 18). The induction of phytoalexin formation is thought to be initiated by the interaction of the fungal conidia with the plant cell. On a molecular level this interaction requires a fungal elicitor molecule which is probably recognized by a receptor in the plant cell wall or membrane. Ultimately, the interaction leads to the activation of genes of the host plant which code for enzymes involved in the biosynthesis of phytoalexins (8, 19, 23). Once formed, the phytoalexins appear to be toxic to the invading pathogen and thereby retard the colonization of the host plant.

As with most other complex biochemical pathways, the defense response in plants is probably not free of biochemical regulation. Thus, there is a growing body of evidence to suggest that elicitation can be both enhanced and suppressed by endogenous and exogenous modulators. For example, xanthan (13) phaseolotoxin (14), and β-(1,3) and β-(1,6) glucans (mycolaminarins) (11, 20) have all been shown to be race specific suppressors of general defense reactions including phytoalexin biosynthesis (20). However, the molecular identities of the specific suppressors in most of these preparations have never been elucidated.

To probe the stimulus-response coupling associated with elicitation, an assay was developed (21) to monitor the initial events occurring in the plant cell upon interaction of the elicitor with the plant cell wall or membrane. This assay utilized membrane potential- and ion selective-fluorescent dyes to monitor the elicitor-induced changes in membrane permeability which occur within minutes of elicitor addition to the cultured plant cells. Since the fluorescence transitions were found to quantitatively correlate with phytoalexin formation, the fluorescence assays were concluded to represent an accurate means of monitoring elicitation.

Using the fluorescence assays described above, we decided to screen several plant metabolites for activity as suppressors or enhancers of elicitation. We report here that citrate, a common component of most plant cell extracts, is a potent inhibitor of elicitation.

MATERIALS AND METHODS

Chemicals. The fluorescent probes, 8-hydroxyppyrene-1,3,6-trisulfonic acid, trisodium salt (pyranine), and bis-(3-propyl-5-oxoisoxazol-4-yl) pentamethine oxol (oxolin VI) were purchased from Molecular Probes, Eugene, OR. Stock solutions of these dyes were prepared (1 mg/ml) in H2O (pyranine) or 50% ethanol (oxolin VI). Media ingredients were reagent grade chemicals and purchased from Sigma Chemical Co. and from Mallinckrodt, Inc. Glyceollin was a gift from Dr. David Kuhn, Purdue University. Cotton phytoalexins were previously isolated and characterized (2, 16). Sodium citrate solutions were adjusted to pH 6.0 and sterilized by membrane filtration (GS 0.2 μm, Millipore).

Received for publication January 27, 1987 and in revised form April 24, 1987

1 Research was supported in part by a grant from Monsanto Company.
2 Permanent address: Institute of Medical Biochemistry, N. Copernicus Medical Academy, 31-304 Krakow, Kepernika 7, Poland.
Plant Cell Cultures. Cell suspension cultures of *Gossypium arboreum* L. Nanking were initiated and maintained as previously described (17). *Glycine max* Merr var Kent cultures were obtained from Dr. Mike Hasegawa, Purdue University and were grown in W-38 medium (15). Cultures were transferred to fresh medium every 7 d.

**Phytalexin Assays.** Phytalexins of cotton cell suspension cultures were determined as described (16). Briefly 1 cm³ of loosely packed cells of a 6-d-old culture was inoculated into 20 ml of medium. After 36 h of incubation, experiments were started with the addition of elicitor preparation and citrate, as indicated. The cells were then cultured as above for an additional 48 h, after which the column was divided or injected with an additional 48 h incubation. The column was harvested after which the methanol extraction was used to determine dry weight (16).

The formation of glyceollin in the medium of soybean cell suspension cultures was measured according to the procedure of Ebel *et al.* (12). Three cm³ of loosely packed cells of a 6-d-old soybean culture were inoculated into 100 ml of fresh medium. After an incubation period of 30 h, experiments were started with the addition of elicitor preparation and citrate, as indicated. Cells were harvested after an additional 40 h incubation and glyceollin was determined (12) in the medium by extraction with 15 ml of ethyl acetate. After separation of the two layers the ethyl acetate was removed under a stream of N₂ and the residue taken up in 100 μl of ethanol (95%). The ethanol solution was quantitatively applied onto a 2 mm thick silica gel thin layer plate. The plate was developed with toluene-chloroform-acetone (40:25:35, v/v) and the spot corresponding to glyceollin, identified by comparison of its Rf value with the migration of authentic glyceollin, extracted with 2 ml ethanol. Glyceollin was measured spectrophotometrically at 285 nm (E₂85 = 10,300, ethanol) after purification on silica gel plates and detection at 254 nm (12). The cell mass residue was used for dry weight determination.

**Formation of Phytalexins in Cotton Seedlings.** *G. arboreum* seeds (500) were warmed in 80°C water for 105 s and quickly chilled in ice water. Floating seeds were discarded and the remaining seeds were allowed to germinate in damp paper towels in the presence of 1 g benomyl/L H₂O. The seeds were maintained for 4 d at 28°C, and either cold shocked for 4 d at 10°C or injected with live *Verticillium dahliae* conidia. The germinated seedlings were severed at the transition zone and the rants were divided into groups of 2.2 g each and placed into 50 ml of 0.02 M potassium phosphate buffer (pH 6.5), containing 0.5% sucrose and 0.1% sodium citrate. Varying amounts of citrate were then added. The pH of the incubation solution was unaffected by the addition of citrate. The subsequent incubations were carried out in 22 × 12 × 7 cm glass pans. The seedlings were then covered with a plastic wrap. After 48 h at 28°C, the seedlings were removed and allowed to stand in 50 ml of absolute ethanol for 15 min. The ethanol was decanted and the seedlings homogenized 3 times in 10 ml of absolute ethanol. The combined ethanol extracts were filtered consecutively through Whatman No. 50 filter paper and a sintered glass, medium porosity filter. The filtrate was evaporated to dryness and taken up in HPLC grade methanol. The solution was filtered (1.2 μm) and diluted to 2 ml. Aliquots were injected onto a 5 m Sperisorb Hexyl column (25 cm × 4.6 mm) in a Hewlett Packard 1090 high pressure liquid chromatograph, equipped with a divide array detector. The column was maintained at 40°C. Elution was accomplished with a gradient of methanol and H₂O each containing 0.1% H₃PO₄. From 0 to 5 min a methanol/H₂O gradient of 20 to 60% was used and from 5 to 30 min a methanol/H₂O gradient of 60 to 100% was employed. The following retention times were recorded, in min: deoxy hemigossypol (dHG), 10.4; hemigossypol (HG), 10.8; deoxy-6-methoxy hemigossypol (dMHG), 11.7; 6-methoxy hemigossypol (MHG), 12.1; gossypol (G), 16.7; 6-methoxy gossypol (MG), 17.2; 6,6'-dimethoxy gossypol (DMG), 17.7; G, MG, and DMG were not adequately resolved and are reported as one peak.

**Elicitor.** The crude elicitor used throughout these experiments was an autoclaved cell wall-membrane preparation of *V. dahliae* 277, as described (21). A typical elicitor preparation contained 70 μg of protein and 134 μg of glucose equivalents per ml (21). Elicitor was stored frozen under sterile conditions. Since the rate of fluorescence decay upon addition of different concentrations of elicitor showed a biphasic response (21), care was taken to use optimum elicitor concentration for all incubations.

**Fluorescence Assay.** The rate of fluorescence change upon addition of elicitor was measured in a Perkin Elmer MPF 44A spectrophotofluorometer as previously described (21) with a few modifications. To 1.2 ml of cells in a 4 ml quartz cuvette was added 2 μl of dye stock solution. When citrate was used in the experiment, the addition of citrate preceded elicitor addition by about 15 s. Changes in fluorescence were recorded continuously and the rate of fluorescence change was calculated from the slope over a region of the scale where a linear decrease in fluorescence was observed (21). Linearity was usually obtained between 80 and 50% of the original fluorescence.

**RESULTS**

The induction of phytalexin biosynthesis by elicitor molecules and the associated membrane changes can be conveniently assayed with fluorescent molecular probes (21). The elicitor-induced changes of the probe's fluorescence occur within minutes after addition of elicitor and presumably correspond to early events in the signal transduction pathway (21). Using two such probes, pyranine, a membrane-impermeant, pH-sensitive fluorescent dye and oxonol, a membrane potential-sensitive dye which partitions into the membrane, elicitation of cotton and soybean cells was monitored. As previously noted, a rapid decrease in fluorescence was observed after a large period of 5 to 7 min following addition of an elicitor preparation from *V. dahliae* (Fig. 1A). Prior addition of 6 mm sodium citrate partially inhibited the response and 30 mm citrate completely eliminated the pyramine transition. A similar experiment using oxonol VI as the molecular probe showed that 6 mm sodium citrate had no effect and 30 mm citrate only partially inhibited the elicitor-induced fluorescence change (Fig. 1B). The sodium citrate concentration required to obtain 50% inhibition (ID₅₀) of the elicitor-induced pyramine transition appeared to be about 2 mm for both *G. arboreum* and *G. max* cells (Fig. 2).

Since the elicitor-induced fluorescence changes of pyramine have been shown to correlate with phytalexin formation in cotton cell suspension cultures (21), the effect of citrate on phytalexin formation in *V. dahliae* elicitor-induced cotton and soybean cell suspension cultures was investigated. When sodium citrate was added to *G. arboreum* cells prior to addition of elicitor, a concentration dependent decrease in phytalexin formation was observed (Fig. 3), confirming the prediction of the fluorescence assay (Fig. 1A). The concentration required for 50% inhibition (about 2 mm) was also similar to that seen above (Fig. 2). However, when the order of elicitor and citrate addition was reversed, i.e. elicitor first and citrate 0.5 to 30 min later, only a minor diminution of the defense response was measured, suggesting the sequence of elicitor and citrate addition is critical to the inhibitory mechanism. Curiously, citrate at ID₅₀ concentrations or less, stimulated cell mass accumulation in the same cell suspension cultures (Fig. 4).

A similar effect of citrate on glyceollin formation in soybean cell suspension cultures was observed. Addition of 660 μl (46.2 μg protein, 88.4 μg glucose equivalent) of an elicitor preparation from *V. dahliae* to a 100 ml culture of *G. max* caused the
accumulation of 11.4 μmol glycine/g dry weight in the medium compared to 0.38 μmol/g dry weight in unstimulated cultures. However, as before, addition of 5 mM citrate prior to elicitation inhibited glycine accumulation by 70%. Furthermore, citrate also increased cell growth in soybean cultures (Fig. 5); however, this increase was not as pronounced as in the cotton cell cultures. At the highest citrate concentration tested (5 mM), 0.38 g dry weight of cell mass accumulated in 100 ml compared to 0.3 g dry weight in 100 ml of untreated cell suspensions.

The inhibitory effect of citrate was specific. Other di- and tricarboxylic hydroxy acids, with the possible exception of malate, did not appreciably inhibit the elicitor-induced fluorescence changes. However, malate at a concentration of 25 mM inhibited elicitation with 57% the potency of citrate (Table I). Addition of

**Fig. 1.** Effect of citrate on elicitor-induced pyranine (A) and oxonol VI (B) fluorescence transitions in soybean cell suspension cultures. Soybean cell suspensions (1.2 ml) were treated with 2 μl of pyranine (A) (pH-sensitive dye) or 2 μl of oxonol VI (B) (membrane potential sensitive dye). Thereafter, the following additions were made: (1) 10 μl of an elicitor preparation from V. dahliae; (2) 6 mM citrate followed about 15 s later by 10 μl of elicitor; (3) 30 mM citrate followed about 15 s later by 10 μl of elicitor; or (4) no additions. Fluorescence was continuously monitored and recorded as described in “Materials and Methods.”

**Fig. 2.** Effect of increasing concentrations of citrate on the elicitor-induced fluorescence transitions of pyranine in cultured cotton (●●●●●) and soybean (X--X) cells. Experimental details as in Figure 1. Arrows indicate the inhibitory dose required for approximately 50% inhibition (ID50).

**Fig. 3.** Elicitor-induced sesquiterpene aldehyde phytoalexin formation in cotton cells as a function of time at varying citrate concentrations. *G. arboreum* cells (1 cm² in 20 ml of medium) were treated with 0.15 ml of elicitor preparation (X--X), 0.15 ml elicitor and 180 μM citrate (●●●●●), 0.15 ml elicitor and 1.8 mM citrate (A--A), or 0.15 ml elicitor and 18 mM citrate (■■■). Citrate was added 5 min before elicitor addition. Control incubations without elicitor or citrate addition were obtained at zero time and after 120 h (○). Sesquiterpene aldehydes were determined in the cell mass by the phloroglucinol method (see “Materials and Methods”).
was the strong in dry weight citrate fluorescence transition.

dahliae 277 amounts on and after 120 phytoalexin incubations of cultured ml of elicitor, (x-x) elicitor 0.2 0.39 E

FIG. 4. Effect of the addition of citrate and crude elicitor on the growth of cultured cotton cells. Experimental details are as in Figure 3. (x-x) elicitor alone, (o-o) 180 μM of citrate followed by 0.15 ml of elicitor, (△-△) 1.8 mM citrate followed by 0.15 ml elicitor, (□-□) 18 mM citrate followed by 0.15 ml of elicitor. The cell mass after phytoalexin extraction was used to measure dry weight. Control incubations without elicitor or citrate addition were obtained at zero time and after 120 h (○).

FIG. 5. Effect of citrate on the elicitor-induced glyceollin formation and on cell growth in soybean cell suspension cultures. Three cm² of cells were inoculated into 100 ml of medium. After 30 h of incubation increasing amounts of citrate were added followed by 0.66 ml of V. dahliae 277 elicitor preparation. Glyceollin (●-●) was determined in the medium after a 40 h incubation. The cell mass was used for dry weight determination (x-x). Control incubations without elicitor and citrate contained 0.38 mmol glyceollin/g dry cells and 0.3 g of cellular dry weight after 40 h of incubation.

the strong Ca²⁺ chelator, EGTA, at a concentration where all Ca²⁺ in the medium would have been chelated, did not inhibit fluorescence transition.

The effect of citrate on the induction of phytoalexin formation was further investigated in 4-d-old G. arboreum seedlings elicited either by cold shock or treatment with V. dahliae. The results

Table I. Inhibition of the Elicitor-Induced Pyranine Fluorescence Transition in G. max var Kent Cells by Various Di- and Tricarboxylic Acids

Soybean cells (1.2 ml) were treated with pyranine and elicitor (V. dahliae 277) (10 μl) with and without various di- and tricarboxylic acids (25 mM) and with EGTA (2.5 mM). Percent inhibition was calculated from the rate of fluorescence change, where the change observed with elicitor alone was taken as 0% inhibition (Fig. 1A, curve 1) and the fluorescence response in the absence of elicitation as “100% inhibition” (Fig. 1A, curve 4).

<table>
<thead>
<tr>
<th>Addition</th>
<th>Inhibition %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elicitor</td>
<td>0</td>
</tr>
<tr>
<td>Elicitor + Na citrate</td>
<td>100</td>
</tr>
<tr>
<td>Elicitor + Na succinate</td>
<td>2.9</td>
</tr>
<tr>
<td>Elicitor + Na tartrate</td>
<td>14.3</td>
</tr>
<tr>
<td>Elicitor + Na maleate</td>
<td>2.9</td>
</tr>
<tr>
<td>Elicitor + Na malate</td>
<td>57.1</td>
</tr>
<tr>
<td>Elicitor + EGTA</td>
<td>0</td>
</tr>
</tbody>
</table>

Table II. Effect of Na Citrate on Phytoalexin Formation in G. arboreum Seedlings

G. arboreum seedlings were either treated with V. dahliae elicitor preparation (experiment 1) or cold shocked for 4 d at 10°C (experiment 2) to induce phytoalexin formation. After 48 h at 28°C the roots were extracted with ethanol and analyzed for sesquiterpene aldehyde concentration as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th>Na Citrate</th>
<th>Phytoalexin Formation</th>
</tr>
</thead>
<tbody>
<tr>
<td>mM</td>
<td>µg/g fresh wt</td>
</tr>
<tr>
<td>Experiment 1</td>
<td>dHG HG dMHG MHG G, MG, DMG</td>
</tr>
<tr>
<td>0</td>
<td>7 14 2 6 290</td>
</tr>
<tr>
<td>0.18</td>
<td>4 10 2 6 253</td>
</tr>
<tr>
<td>0.36</td>
<td>4 10 3 9 246</td>
</tr>
<tr>
<td>0.72</td>
<td>5 9 3 7 210</td>
</tr>
<tr>
<td>1.44</td>
<td>4 8 2 6 189</td>
</tr>
<tr>
<td>Experiment 2</td>
<td>dHG HG dMHG MHG G, MG, DMG</td>
</tr>
<tr>
<td>0</td>
<td>51 55 12 19 236</td>
</tr>
<tr>
<td>1.8</td>
<td>12 14 8 17 149</td>
</tr>
<tr>
<td>9.0</td>
<td>— — 6 7 39</td>
</tr>
<tr>
<td>18.0</td>
<td>— 4 — 4 13</td>
</tr>
<tr>
<td>180.0</td>
<td>— 2 — 1 9</td>
</tr>
</tbody>
</table>

*a Not detectable. G, gossypol; MG, 6-methoxy gossypol; DMG, 6,6’ dimethoxy gossypol; MHG, 6-methoxy hemigossypol; dMHG, deoxy-6-methoxy hemigossypol; HG, hemigossypol; dHG, deoxy-hemigossypol.

obtained (Table II) show that the fungal pathogen-elicited formation of the major cotton phytoalexins, (HG, G, MG, DMG) was reduced at citrate concentrations of 0.18 to 1.44 mM. At higher concentrations of citrate and in cold-shocked seedlings the inhibitory effect was considerably more pronounced (Table II).

DISCUSSION

The elicitation of phytoalexin formation in plants has been attributed to a variety of substances. Some of these have been found to be highly specific pathogen- or plant cell wall-derived molecules, while others may be simply general cellular perturbants (9). Specific inhibition of the plant cell response to elicitor molecules has not been reported although a number of biological extracts and buffers have been found to partially attenuate elicitation (10, 20, 22, 24). We have found that phys-
iological concentrations of citrate, a normal plant metabolite, can effectively inhibit elicitation.

The selectivity of the citrate effect is demonstrated by the relative impotence of similar di- and tricarboxylic hydroxy acids with the possible exception of malate (Table I). Furthermore, as shown in Figure 1, citrate was found to block only the pyranine fluorescence transition and not the transition of oxonol, suggesting proton fluxes are specifically inhibited without significantly altering the changes in membrane potential deriving from other ion fluxes. Thus, the oxonol transition was only altered at very high, toxic citrate concentrations where cotton cell growth was inhibited (Fig. 4). Such toxicity can be excluded as the cause of the observed suppression of elicitation at lower citrate concentrations, since 1.8 mM citrate actually increased cotton cell growth over and above control values (Fig. 4) even though it inhibited both the elicitor-induced fluorescence changes as well as phytoalexin formation. Similarly, oxonol cell growth in culture was increased by low concentrations of citrate (Fig. 5). Finally, the specificity of the citrate inhibition can be inferred by the observation that addition of citrate 30 s to 15 min after addition of elicitor nearly obliterates the inhibitory effect, i.e. the order of elicitor and suppressor (citrate) addition is apparently very important.

Although the molecular mechanism by which citrate inhibits elicitor-induced phytoalexin formation is at present unclear, certain observations are noteworthy. First, the inhibition most likely occurs at the cell membrane, since (a) the molecular probes which first detect the inhibition reside at this site (5–7, 25) and (b) as pointed out above, addition of citrate after treatment with elicitor has diminished inhibitory effect on both the pyranine fluorescence transition and on phytoalexin formation. Second, since the oxonol-detected, elicitor-dependent events in the membrane are largely unaffected by citrate, it can be suggested that citrate blocks only one of several elicitor stimulated changes. By the same argument, it is clear that oxonol and pyranine report on different elicitor-stimulated events. Whether these citrate-resistant events involve elements of a signal transduction sequence leading to the biosynthesis of other defense products cannot be concluded from the data. However, citrate apparently does not act as a Ca\(^{2+}\) chelator since incubations with 0 to 5 mM EGTA or EDTA showed no inhibition of fluorescence transition. Similarly, simple Ca\(^{2+}\) chelation by citrate as the inhibitory mechanism is not likely from the observation that malate showed 57% inhibition of elicitor induced fluorescence transition yet is equally effective in binding Ca\(^{2+}\) as tartrate and succinate, which showed no inhibition, and is a poorer Ca\(^{2+}\) chelator than maleate, which did not inhibit (Table I). Finally, since citrate was effective in inhibiting elicitation in whole seedlings by both *V. dahliae* and cold-shock, it is probable that both stimuli, biotic and abiotic, depend on a common, citrate sensitive pathway in promoting the defense response in plants.

**LITERATURE CITED**

5. BELL DH, LK PATTERSON, JM GOULD 1983 Transmembrane pH gradients and functional heterogeneity in reconstituted vesicle systems. Biochim Biophys Acta 752: 368–375
15. HASEGAWA PM, R BRENNIS, A HANDA 1980 Growth characteristics of NACL-selected and non-selected cells of *Nicotiana tabacum* L. Plant Cell Physiol 21: 1347–1355