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Binding of the phytotoxin zinniol stimulates the entry of calcium into plant protoplasts

(receptor/calcium-channel agonist)

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ABSTRACT **Zinniol [1,2-bis(hydroxymethyl)-3-methoxy-4-methyl-5-(3-methyl-2-butenyloxy)benzene], a toxin produced by fungi of the *Alternaria* group, causes symptoms in plants that resemble those induced by the fungi. The phytotoxin binds to carrot protoplasts and isolated membranes in a saturable and reversible manner. Receptor occupancy stimulates entry of calcium into protoplasts. Zinniol can partially reverse the effects and binding of the calcium-channel blockers desmethoxyverapamil and bepridil. Selected cell lines that are insensitive to zinniol lose part of their binding capacity and sensitivity to the action of the agonist-like compound but are still able to bind calcium-channel blockers. We conclude that zinniol acts on calcium entry but that the targets of the toxin and of calcium-channel blockers are dissimilar, suggesting the occurrence of sites affected both by zinniol and by channel blockers and of sites affected only by zinniol.**

The conversion of a stimulus into the appropriate biological responses involves a cascade of events starting with the primary recognition of the signal by membrane receptors and the subsequent increase of intracellular messenger concentrations. Such a general framework is well documented in animal cells (1) where toxins have been shown to interfere with regulatory compounds by binding to their specific receptors, disturbing the concentrations of second messengers (2, 3). Plants are obviously able to respond to changes in the environment in a very efficient way (4). Membranes isolated from plant cells bind a number of physiologically important ligands including plant growth substances (e.g., auxin and abscisic acid), herbicides (5, 6), toxins (7), and fungal phytoalexin elicitor (8). However, in most cases, the link between receptor occupancy and the intracellular signaling systems remains to be investigated.

To date, chemical stimulus–biochemical coupling has been established in a limited number of examples where phytotoxins are involved. Thus, fusicoccin binds to a component of the plasmalemma-bound ATPase and activates the proton-extrusion system (9). Similarly, tentoxin inhibits photophosphorylation on binding to the choloroplast coupling factor CF_1 (10) and helminthosporosides lead to membrane depolarization by interacting with the plasmalemma (11).

Previous work has shown that zinniol [1,2-bis(hydroxymethyl)-3-methoxy-4-methyl-5-(3-methyl-2-butenyloxy)-benzene] exhibits a very broad phytotoxic spectrum (12) and promotes, by itself, symptoms resembling those induced by fungi of the *Alternaria* group (13). In this report, we present evidence for the specific binding of zinniol to protoplasts and membranes from zinniol-sensitive cell lines. Receptor occupancy stimulates the entry of calcium into protoplasts and partially reverses the inhibitory effects of calcium-channel blockers. Cell lines resistant to zinniol do not respond in this manner but are still able to bind calcium-channel blockers and to be sensitive to their effects. Therefore, zinniol may act specifically on a particular class of plant calcium channel.

MATERIALS AND METHODS

Cell Culture and Selection. Friable carrot cell aggregates were grown on solid medium as described (14). Zinniol-insensitive cell lines, generously supplied by Société Clause (Paris), were isolated as follows. Carrot calluses were plated onto Petri dishes (8 cm in diameter) containing solid medium supplemented with 1.16 mM zinniol. After 2 weeks, surviving colonies were subcultured on medium containing 0.5 mM zinniol for 53 days. Then the selected lines were grown on the standard medium and periodically checked for their insensitivity to zinniol.

Protoplast Preparation. In a typical experiment, 1 g of 10-day-old cells was transferred into an 8-cm Petri dish containing 700 mM mannitol, 2% Caylase 345, 0.1% pectolyase Y23, 1 mM iodoacetamide, 0.1 mM phenylmethylsulfonyl fluoride, and 0.01 mM pepstatin A in 20 ml of 25 mM Mes/Tris buffer (pH 5.5). The suspension was incubated at 36°C with gentle shaking (44 rpm) for 90 min. Then the suspension was filtered through 25-μm nylon mesh and the filtrate was centrifuged at 500 × *g* for 3 min. The pellet, containing crude protoplasts, was resuspended in 2 ml of 25 mM Hepes/KOH buffer (pH 6.7) containing 700 mM mannitol and 20% Ficoll 400 (buffer A). On top of the protoplast suspension, a Ficoll gradient was prepared by layering successively 2 ml of buffer A in 10% Ficoll 400 and 1 ml of buffer A without Ficoll. The discontinuous gradient was centrifuged at 500 × *g* for 30 min at 4°C. The purified protoplasts were obtained at the 0%/10% Ficoll interface and washed by centrifugation at 500 × *g* for 3 min with 4 ml of buffer A without Ficoll. The pellet was resuspended in buffer A without Ficoll, at a concentration of 3–4 × 10^6 protoplasts per ml.

Cell Microsome Preparation. Cell microsomes were prepared and stored as described (15); 8 mg of microsome protein was obtained from 25 g of fresh carrot cells.

Binding Experiments. In routine assays, protoplasts (3 × 10^6) or microsomes (20 μg) were incubated at 20°C in 1 ml of 25 mM Hepes/KOH buffer (pH 7.5) containing 700 mM mannitol, 0.01% bovine serum albumin, and 5 mM KCl. The mixture was supplemented with either 4.5 nM [*O*^{3}-*methyl*-^{3}H]zinniol (10^5 dpm) or 6.3 nM [*N-methyl*-^{3}H]desmethoxyverapamil {(−)-[^{3}H]D 888; 1.1 × 10^6 dpm}. Where indicated, 100-μl (protoplasts) or 400-μl (membranes) aliquots of the incubation mixture were removed and filtered under reduced pressure through Whatman GF/C filters, and the filters were

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rapidly washed three times with 2 ml of ice-cold 20 mM Tris/HCl buffer (pH 7.5) containing 500 mM mannitol. The radioactivity remaining on the filters was measured in a liquid scintillation spectrometer. Experiments were done in triplicate and at least three times each independently. Nonspecific binding to microsomes was measured in the presence of 20 μM unlabeled zinniol or 50 μM calcium-channel blockers.

Calcium Uptake. Protoplasts (10^6 per ml) were preincubated in 25 mM Hepes/KOH buffer (pH 6.7) with 700 mM mannitol and 5 mM KCl for 60 min at 20°C in the presence of the appropriate compound. Calcium uptake was initiated by adding 0.1 mM $CaCl_2$ and 0.7 μCi of $^{45}CaCl_2$ (1 μCi = 37 kBq). At the indicated times, 300-μl aliquots were filtered through HAWP Millipore filters and the filters were washed three times with 2 ml of ice-cold 20 mM Tris/HCl buffer (pH 7.5) containing 100 mM $MgCl_2$ and 500 mM mannitol. The radioactivity remaining on the filters was measured in a liquid scintillation spectrometer.

Chemicals. (−)-[^{3}H]D 888 (67 Ci/mmol) was from Amersham and $^{45}CaCl_2$ was from New England Nuclear. (−)-Bepridil was from Centre d'Etude et de Recherche Médicale (Riom, France). Caylase 345 (a protease-poor cellulase) was from Société Caylase (Toulouse, France). Pectolyase Y23 was from Seishin Pharmaceutical Co. (Tokyo). Unlabeled zinniol was isolated as described elsewhere (13).

[*O*^{3}-*methyl*-^{3}H]Zinniol was synthesized by a two-step procedure (16). Methyl 2-formyl-3-hydroxy-4-methyl-5-(3-methyl-2-butenyloxy)benzoate (kindly supplied by J. A. Martin, Roche Products, Ltd., Welwyn Garden City, U.K.) was reduced with $LiAlH_4$. The reduced compound (35 μg) was isolated and methylated with 25 mCi of CH_3I (Amersham; 9.9 Ci/mmol) in dry acetone in the presence of 2 mg K_2CO_3. The labeled zinniol thus obtained was purified by reverse-phase HPLC and the purity was checked by cochromatography with an authentic sample of the phytotoxin.

All other chemicals were analytical grade.

RESULTS

Binding of [^{3}H]Zinniol to Protoplasts and Microsomes. The binding of [^{3}H]zinniol to protoplasts was time-dependent and reached a plateau corresponding to 190 fmol (sensitive cell lines) or 60 fmol (resistant cell lines) of zinniol per 10^6 protoplasts after a 60-min incubation (Fig. 1*A*). Addition of excess unlabeled ligand (10 μM) to the equilibrated systems led to a rapid loss in radioactivity associated with the protoplasts. Such reversibility indicates that virtually all the labeled zinniol was displaced within 60 min and suggests that the process was specific (the nonspecific component, which was subtracted systematically, represented <15 fmol). The zinniol receptors appeared to be most accessible when the protoplasts were suspended in a medium containing KCl (5–100 mM). The resistant cell lines had lost an important part of their ability to bind the phytotoxin. From the best fitted curve, plotted according to McPherson (17), two values for the equilibrium dissociation constant (K_d) were determined,

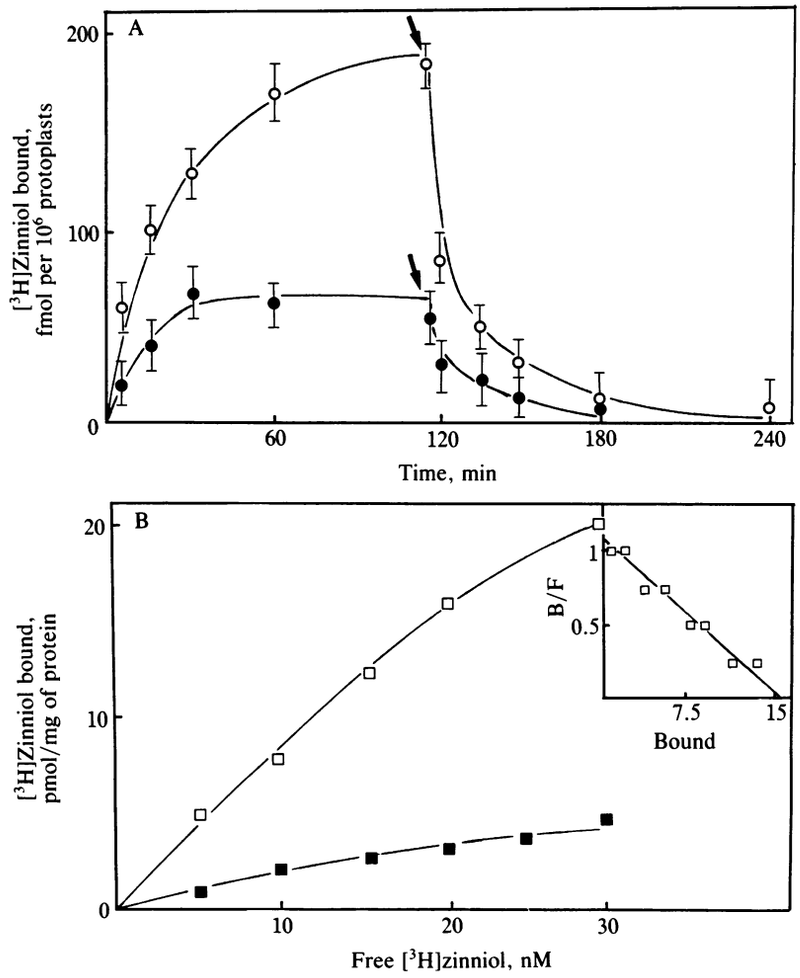

FIG. 1. Binding of [^{3}H]zinniol to carrot protoplasts and microsomes. (*A*) Kinetics of binding to carrot protoplasts of sensitive (○) or resistant (●) cell lines. Arrows indicate the addition of 10 μM unlabeled zinniol and the lines represent the best fit (17). (*B*) Equilibrium binding of [^{3}H]zinniol to microsomes from sensitive cell lines in the absence (□) or the presence (■) of 20 μM unlabeled zinniol. Time of binding was 1 hr. (*Inset*) Scatchard plot for the specific [^{3}H]zinniol-binding component. Bound, pmol/mg of protein; bound/free (B/F), ml/mg.

corresponding to a low-affinity site (K_d = 103 mM) and a high-affinity site (K_d = 5.17 nM).

Fig. 1*B* shows equilibrium binding of [^{3}H]zinniol to carrot microsomes. For sensitive cell lines, the specific binding component is large as compared to the nonspecific binding component. The Scatchard plot shows only a single type of binding site with K_d = 15 nM (3-fold higher than for protoplasts) and a maximal binding capacity B_{max} = 16.2 pmol/mg of protein (Fig. 1*B Inset*). For insensitive cell lines, the specific binding component is only slightly greater than the nonspecific one, so no accurate measurements could be made (data not shown).

Significance of Site Occupancy. The significance of site occupancy was examined by tentative correlation with other plasmalemma activities. Zinniol had no effect on either the redox pump or the proton-extruding system of intact protoplasts and did not modify the Ca^{2+}-ATPase activity of microsomes (data not shown). In contrast, the addition of the phytotoxin to carrot protoplasts resulted in a marked stimulation of calcium influx (Fig. 2*A*). With sensitive lines, zinniol acted maximally at concentrations of 0.1–1 μM, which provoked a 60% increase of influx over the control value, with half-maximal stimulation at 30 nM (Fig. 2*B*). Higher concentrations led to less efficient stimulation that was essentially nullified at 0.1 mM. In contrast, up to 0.1 μM zinniol had no effect on resistant cell lines, and the optimal concentration was >0.1 mM.

Interaction Between Zinniol and Calcium-Channel Blockers. Since calcium-channel blockers such as (−)-D 888 and (−)-bepridil inhibit calcium entry into protoplasts and bind to plant membrane preparations (15, 18), we studied possible competition between these drugs and zinniol. (−)-Bepridil inhibited the binding of (−)-[^{3}H]D 888 to protoplasts (Fig. 3*A*) and to membranes (Fig. 3*B* and ref. 15) of both cell lines. In contrast, zinniol inhibited the binding of the channel blocker in sensitive lines by 50% but was without effect in the resistant cell lines. Moreover, the effect of zinniol on calcium uptake by sensitive protoplasts was inhibited in a dose-dependent manner by bepridil (Fig. 4).

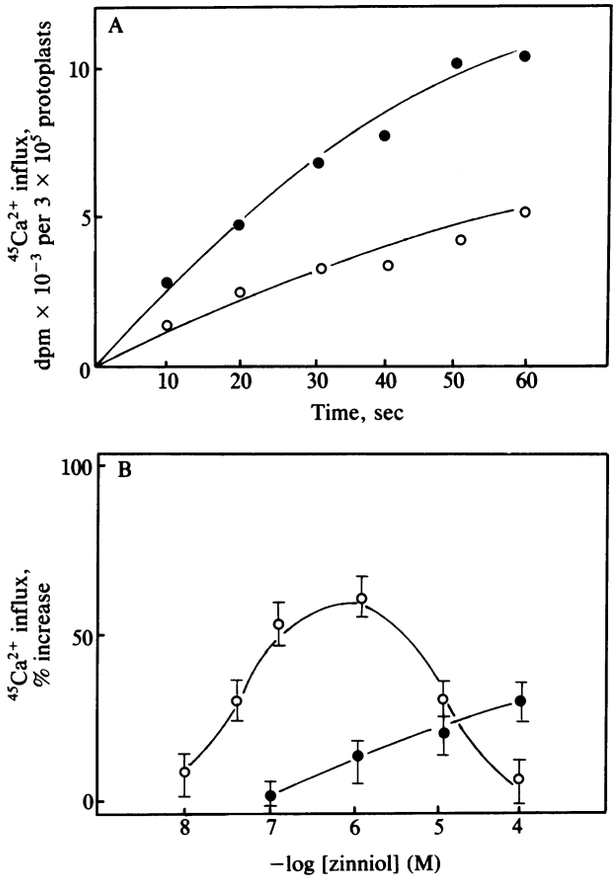

FIG. 2. $^{45}Ca^{2+}$ influx into carrot protoplasts. (*A*) Time course of $^{45}Ca^{2+}$ influx in the absence (○) or presence (●) of 1 μM zinniol. Only data obtained with sensitive cell lines are shown; zinniol has a weak effect on calcium entry into resistant cells at the concentrations used, whereas the overall calcium influx is similar for the two cell lines. (*B*) Effect of zinniol on $^{45}Ca^{2+}$ influx in sensitive (○) and resistant (●) cell lines. Time of $^{45}Ca^{2+}$ influx was 45 sec.

DISCUSSION

This paper describes the binding of zinniol to intact protoplasts and membrane preparations in a reversible and saturable manner. The dissociation constant K_d was higher for membrane preparations than for protoplasts, presumably because the use of crude microsomes led to the dilution of "active" membranes. However, the data obtained are consistent with the existence of a zinniol receptor in carrot cells. Membranes from cell lines selected for resistance to zinniol toxin have a lower affinity for the ligand. Occupancy of the receptors results in the stimulation of calcium uptake, but the resistant cell lines are several orders of magnitude less sensitive (Fig. 2). We conclude that a direct link exists between the activating properties of zinniol and its binding to the plasmalemma of the protoplasts.

In both zinniol-resistant and zinniol-sensitive cell lines, compounds of the verapamil type have been shown to block calcium channels specifically and thereby inhibit calcium

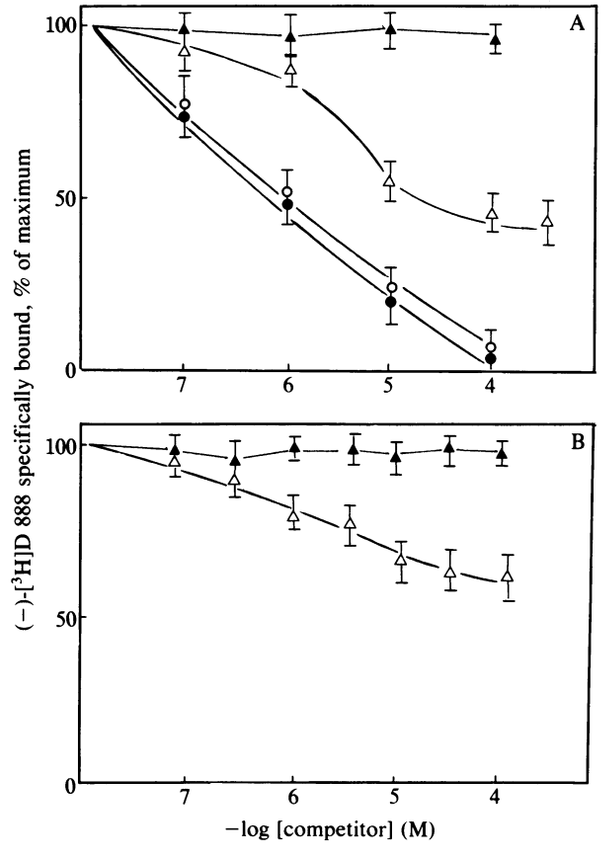

FIG. 3. Equilibrium binding of (−)-[^{3}H]D 888 to carrot protoplasts (*A*) and microsomes (*B*) and its displacement by increasing concentrations of bepridil or zinniol. After (−)-[^{3}H]D 888 was allowed to bind for 1 hr, samples were incubated with bepridil or zinniol for 1 hr. ○, Sensitive lines, displacement by bepridil; ●, resistant lines, displacement by bepridil; △, sensitive lines, displacement by zinniol; ▲, resistant lines, displacement by zinniol.

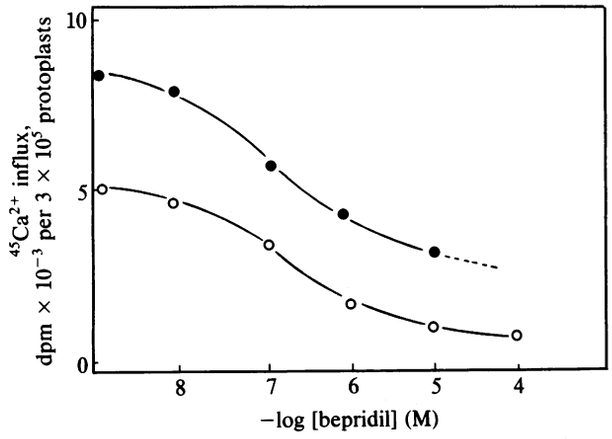

FIG. 4. Effect of bepridil on calcium influx into zinniol-sensitive protoplasts in the absence (○) or presence (●) of 1 μM zinniol. Time of $^{45}Ca^{2+}$ influx was 45 sec.

entry into the protoplasts (15, 18). Since zinniol acts in the converse manner, it may be accordingly considered as a calcium-channel agonist. Consistently, at least in sensitive cell lines, its efficacy is high when compared to that of the blockers. Thus, 30 nM zinniol elicits a 30% activation over control (50% of the maximal effect), whereas equivalent concentrations of inhibitors give rise to no significant modifications (15). Such a high affinity/efficacy and the existence of an optimal concentration have been reported for animal systems (19, 20). However, the self-inhibiting effects of agonists are not clearly understood (21).

The relationship between zinniol and the channel blockers seems complex: (*i*) the ability of membranes to bind antagonists [B_{max} = 120 pmol/mg of protein (15)] is 7.5 times higher than for the toxin (16 pmol/mg of protein); (*ii*) cell lines that have been selected for resistance to zinniol lose only a part of their ability to bind calcium-channel blockers but 80% of their capacity to interact with zinniol; and (*iii*) zinniol does not compete with (−)-D 888 in resistant cell lines and elicits only 50% inhibition with sensitive cell lines. These data show that the targets of the toxin and calcium-channel blockers are not exactly the same. Rather, they suggest the occurrence of two distinct sites; one site would be common to the two compounds and may be modified on habituation of the cells to zinniol, and the other site would be more specific to channel blockers. Whereas the definitive answer will be known only after the purification of the putative receptors, a possible explanation of the competition between zinniol and (−)-D 888 stems from structural considerations. Zinniol and phenylalkylamine-type inhibitors such as (−)-D 888 have similar aromatic moieties that may recognize identical structures. It is known that subtle changes in the substitution of the same basic structure may induce opposite effects in animal systems. Particularly relevant is the example of the potent channel modulators of the dihydropyridine type. For example, nifedipine is a very efficient calcium-channel blocker, whereas Bay K 8644, which is chemically very similar, acts as an agonist (19, 20).

From a more general point of view, it is now accepted that calcium controls various processes in plants, including intracellular organization (22) and various enzyme activities (4) in a direct or indirect manner. In this way, calcium is a second messenger and limited changes in its movements through membranes (especially plasmalemma) are of the utmost importance in transducing the effect of stimuli (for review see ref. 23). Therefore, the stimulatory effects of zinniol on calcium influx may induce a nonregulated increase in cellular calcium. Such a perturbation may have a lethal effect and reflect the early expression of the interaction between the host and a phytopathogenic fungus. The data presented here suggest the possibility of using phytotoxins to look for potential natural agonists or antagonists for ionic channels. Such an approach is frequently used to investigate sodium and calcium channels in animal systems (2, 24). Zinniol may have a very large action spectrum because its molecular target interferes with the functioning of the calcium channels that presumably exist in many different cells. For example, we have found that membrane preparations from the melon (*Cucumis melo* var. Cantaloup Charentais), a plant sensitive to zinniol, bind the phytotoxin and that zinniol can partially displace (−)-D 888 from its receptor (unpublished data).

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