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Communication

Fusicoccin Activity and Binding in *Arabidopsis thaliana*[1]

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RICHARD G. STOUT
Department of Biology, Montana State University, Bozeman, Montana 59717

ABSTRACT

I have examined the activity and specific binding of the phytotoxin fusicoccin using *Arabidopsis thaliana* L. Col-O. Fusicoccin (10 micromolar) stimulates both proton extrusion and enlargement in isolated *Arabidopsis* leaf discs. Radiolabeled fusicoccin specifically binds to membranes (13,000 to 100,000*g* subcellular fraction) from cultured cells of *Arabidopsis*. The specific binding of this phytotoxin to putative receptor sites in *Arabidopsis* membranes is both pH-sensitive (pH optimum = 5.5 to 6.0) and heat-labile (10 min at 70°C). The apparent dissociation constant for the specific binding at 20°C is approximately 1.3×10^{-8} molar. The results of this study are in general agreement with previous reports of fusicoccin binding and activity in other plant species.

Fusicoccin, a fungal phytotoxin produced by *Fusicoccum amygdali*, affects a wide variety of physiological processes in higher plants (7). Evidence from both *in vivo* and *in vitro* experiments supports the hypothesis that FC[2] primarily acts by stimulating the activity of ATPase proton pumps in the plasma membrane of plant cells (7, 9, 10). The putative receptor site for FC is not, however, the ATPase, but another protein located in the plant plasma membrane (13, 14). Recent evidence suggests that FC may also cause a blockage of a K^+ efflux channel in the plasma membrane in addition to stimulating the proton pump (3). Thus, it appears that the binding of this toxin to its receptor may affect at least two transport mechanisms in the plasma membrane of higher plants. This supports the idea that the target site for FC is an important regulatory or signal-transducing protein in the plant cell-surface membrane.

Much information regarding the properties of mammalian cell surface membrane proteins has been gained by obtaining the primary structure through sequencing the DNA coding for these polypeptides. Isolating the gene for the FC binding protein in higher plants will be greatly facilitated by using *Arabidopsis thaliana*, a flowering plant with genetic characteristics that make it an excellent organism for experimental molecular genetics (8). As an initial step toward this goal, I have examined the activity and specific binding of FC in *Arabidopsis*. This is the first report, as far as I'm aware, that FC is active and that a binding site for FC is detectable in *Arabidopsis*.

MATERIALS AND METHODS

Plant Material. Seeds of *Arabidopsis thaliana* L. Col-O (*Arabidopsis* Information Service, Botanisches Institut, J. W. Goethe-Universitat, Frankfurt am Main 11, West Germany) were grown in a mixture of fine vermiculite, perlite, and sphagnum (1/1/1) and were irrigated with mineral nutrients (12). Plants were grown at 18 to 23°C under fluorescent light (120 μE m^{-2} s^{-1}) with an alternating cycle of 16 h light and 8 h dark.

Cultured cells of *A. thaliana* L. Col-O were obtained as described by Feldman and Marks (5). Briefly, leaf explants from sterile, 6-week-old plants, grown under continuous fluorescent light (200 μE m^{-2} s^{-1} L) at 18 to 20°C, were placed in 125-mL Elenmeyer flasks containing liquid medium (CIM). After 1 week in shake culture, the leaf explants were transferred to solid medium (0.15/5, SIM) and grown for 2 to 3 weeks at 20°C under fluorescent illumination (120 μE m^{-2} s^{-1}) with an alternating cycle of 16 h light and 8 h dark.

Fusicoccin Bioassay. Discs (5.5 mm diameter) were obtained from the leaves of 4- to 6-week-old *Arabidopsis* plants using a hole punch. The leaf discs were preincubated for 1 h in solution A, which contained 1% sucrose (w/v) and 1 mM KCl, at 18 to 20°C on an orbital shaker (80 rpm). After preincubation, leaf discs were transferred to 10-mL beaker cups containing 2 mL of fresh solution A (5 discs per cup), and FC (final concentration = 10^{-5} M) or an equivalent amount of ethanol (final concentration = 0.01%) was added. The pH of the solution was determined using a pH meter (Orion Research model 611) equipped with a semimicro electrode (Orion catalog No. 91-03). The initial pH was adjusted to approximately 7.5 with dilute HCl. The leaf disks were briefly (5 min) vacuum infiltrated with the incubation medium, and the beaker cups were covered with parafilm and incubated overnight (16 h) at 18 to 20°C on an orbital shaker (80 rpm). After overnight incubation, the pH of the solution was determined and the diameter of each disc was measured with the aid of a dissecting microscope.

Preparation of Membrane Fraction. A crude membrane fraction was prepared by using 10 to 15 g of callus material from cultured *Arabidopsis* (see above). All steps were carried out at 5°C. The callus material was chopped into small (2–3 mm) pieces with a razor blade in a mortar containing 40 mL of grinding medium consisting of 250 mM sucrose, 10% glycerol, 50 mM Tris-HCl (pH 7.8), 1 mM ethylenediaminetetraacetic acid, 0.2 mM phenylmethylsulfonyl fluoride, and 1 mm dithiothreitol. The callus pieces were ground using mortar and pestle for about 5 min, and the homogenate was strained through four layers of cheesecloth. The filtered homogenate was centrifuged at 13,000 *g* for 10 min, and the resulting supernatant was then centrifuged at 100,000*g* for 30 min. The 100,000*g* pellet was resuspended in 2 mL of the grinding medium (1–2 mg of protein/mL). This crude membrane fraction was stored at −70°C with no significant loss of specific FC-binding capacity after up to three freeze-thaw cycles.

Fusicoccin Binding Assay. Binding assays were performed using [^{3}H]FC (130 TBq/mmol; a gift from Prof. R. E. Cleland). Dihydrofusicoccin is known to have the same effect on plant

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[2]Abbreviations: FC, fusicoccin; [^{3}H]-FC, tritium-labeled dihydrofusicoccin; K_d, dissociation constant; N, concentration of binding sites.

Table I. *Effect of Fusicoccin on Acidification and Growth Using Arabidopsis Leaf Discs*

Five leaf discs from nonflowering *Arabidopsis* plants were incubated for 16 h in 2 mL of an unbuffered solution consisting of 1% sucrose and 1 mM KCl in the presence or absence of 10^{-5} M FC. Mean values with standard error (pH, $n = 3$; leaf disc area, $n = 15$).

Treatment	pH		Leaf Disc Area	
	Initial	Final	Initial	Final
			mm^2	
Control	7.5	7.4 ± 0.1	23.8	34.7 ± 1.4
+ FC	7.5	6.4 ± 0.2	23.8	40.2 ± 1.5

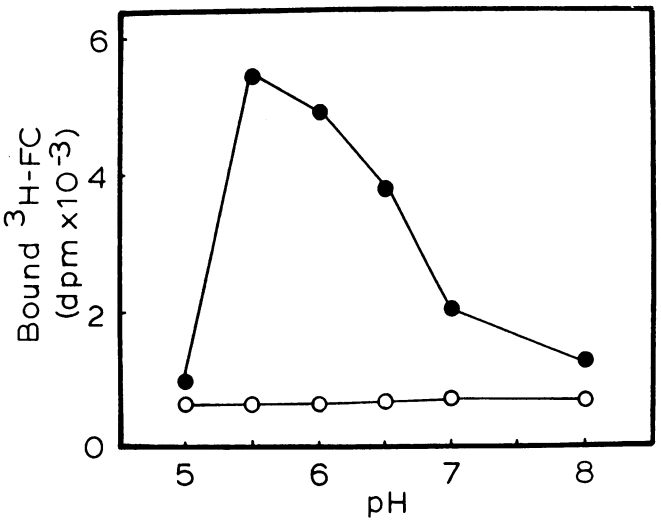

FIG. 1. Effect of pH on [^{3}H]-FC binding to *Arabidopsis* membranes. Identical aliquots (50 μg protein) of a subcellular membrane fraction from *Arabidopsis* were incubated at 20°C for 2 h with 10^{-9} M [^{3}H]-FC (23,400 dpm) in the presence and absence of 10^{-5} M unlabeled FC. The pH of the FC binding medium (see "Materials and Methods") was buffered with 10 mM Mes, adjusted to the pH values shown by adding solid Tris. The amount of radioactivity bound in the presence of excess unlabeled FC, which is known as the nonspecifically bound [^{3}H]-FC (○), was subtracted from the total value to yield the specifically bound counts (●).

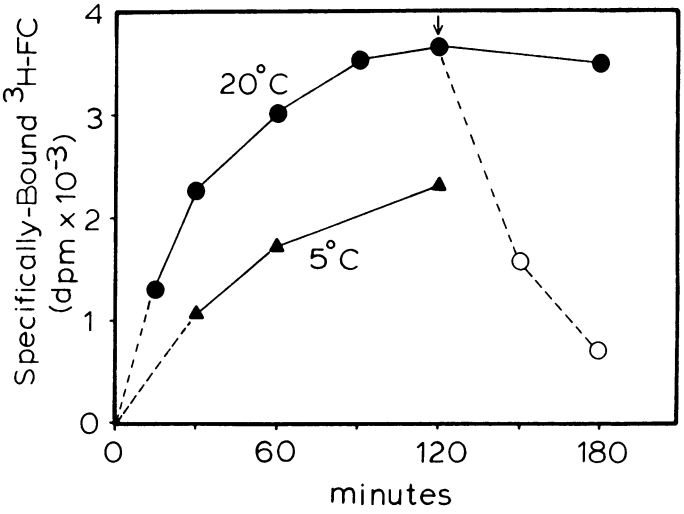

FIG. 2. Time-course of specific [^{3}H]-FC binding to *Arabidopsis* membranes at 20°C (●) and 5°C (▲). At zero time [^{3}H]-FC (final concentration = 10^{-9} M) was added to identical aliquots of *Arabidopsis* membrane material (35 μg protein) in the presence and absence of 10^{-5} M unlabeled FC. At the times shown, membranes were rapidly pelleted at 5°C (10-min centrifugation at 100,000*g*). Nonspecific binding (approximately 400 dpm) was not time-dependent and was subtracted from all radioactivity measurements to yield the specifically bound counts shown above. Arrow indicates time at which 10^{-5} M FC was added to several samples. Radioactivity remaining specifically bound to the membranes after a 30- and 60-min chase with the unlabeled FC is indicated by the open circles (○).

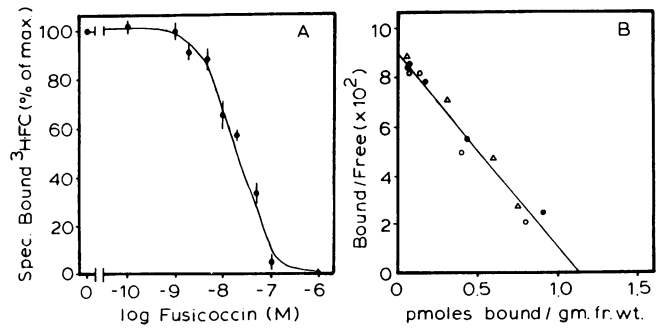

FIG. 3. Saturation kinetics of [^{3}H]-FC binding. A, *Arabidopsis* membranes were incubated for 2 h at 20°C with a constant amount of 10^{-9} M [^{3}H]-FC and increasing concentrations of unlabeled fusicoccin. Nonspecifically bound dpms (in presence of 10^{-5} M FC) were subtracted from total dpms bound to yield specifically bound dpms. The value for 100% equaled about 3500 dpm (approximately 10^5 dpms/mg protein). Data presented is the mean, with standard error, of three separate experiments. B, Scatchard plot of data of A. The different symbols (○, ●, △) represent values from three separate experiments.

tissues as fusicoccin (1, 4). Membrane material (35–50 μg protein) was incubated in binding medium (final volume of 0.3 mL) consisting of 50 mM sucrose, 5 mM $MgSO_4$, buffered with either 10 mM citrate (pH 5.5) or 10 mM Mes-Tris (pH 5–8), 10^{-9} M [^{3}H]-FC (23,400 dpm), in the presence and absence of 10,000-fold excess (10^{-5} M) unlabeled FC. Unless otherwise noted, the membranes were incubated at 20°C for 2 h in the presence of [^{3}H]-FC and then centrifuged at 100,000*g* for 10 min at 5°C. After the 100,000*g* supernatant was withdrawn, the pellet was gently rinsed with 1 mL of distilled H_2O and resuspended in 0.5 mL of distilled H_2O with the aid of a sonicator (E/MC Ultrasonic Cleaner model 250). An aliquot (0.25 mL) of the resuspended membranes was transferred to vials containing 5 mL of scintillation cocktail (Scintiverse, Fisher Scientific) and counted in a Packard scintillation counter.

The amount of specifically bound [^{3}H]-FC was determined by subtracting the dpms associated with the membrane pellet in the presence of 10,000-fold excess unlabeled FC (10^{-5} M) from the value obtained in the absence of unlabeled FC. The [^{3}H]-FC binding data presented is the average of triplicate samples. Each experiment was repeated at least twice using different membrane preparations.

Scatchard Analysis of Binding Data. To determine the saturation kinetics of [^{3}H]-FC binding, data were analyzed according to Scatchard (11). Bound/free values were calculated as ([^{3}H]-FC specifically bound)/(total [^{3}H]-FC added minus the total [^{3}H]-FC bound, *i.e.* specifically and nonspecifically). For each point, the value for pmol bound per g fresh weight was calculated by dividing the specifically bound [^{3}H]-FC (adjusted to per g fresh weight of *Arabidopsis* callus tissue) by the specific radioactivity of FC of that treatment (specific radioactivity decreases with increasing amount of unlabeled ligand). The apparent dissociation constant (K_d) is equal to (−1/slope) and the concentration of binding sites (N) is equal to the intercept on the abscissa.

Protein. Protein content of *Arabidopsis* membranes was determined as previously described (13).

RESULTS AND DISCUSSION

Fusicoccin is known to stimulate cell enlargement and proton extrusion in many higher plants (7). To determine whether or not FC elicits similar physiological responses in *Arabidopsis*, I incubated leaf discs in the presence and absence of 10^{-5} M FC and measured both the pH of the bathing medium and leaf discs diameter. As shown in Table I, FC stimulates both growth and acidification in *Arabidopsis* leaf discs. The FC-induced acidification observed here presumably resulted primarily from a stimulation of plasma membrane ATPase proton pumps (9, 10). The FC stimulation of leaf disc enlargement was likely due to cell wall acidification (7). This adds *Arabidopsis* to the list of plant species responsive to FC and further strengthens the idea that the target site for this toxin is universal in higher plants.

To characterize the nature of FC binding in *Arabidopsis*, a crude membrane fraction was isolated from cultured cells, and the specific binding of [^{3}H]-FC to this subcellular fraction was examined. I used cultured cells instead of leaf tissue because I could obtain more tissue faster by growing leaf explants in culture than by growing plants from seed. Since specific FC binding has been shown to occur primarily in plasma membrane-enriched material from a number of different plant species (2, 4, 6, 13, 14), [^{3}H]-FC binding to soluble material from the *Arabidopsis* homogenate was not investigated. The effect of pH on [^{3}H]-FC binding is shown in Figure 1. Specific binding was optimum at pH 5.5 to 6.0, and there was no significant effect of pH on nonspecific binding. Subsequent binding assays were performed at pH 5.5.

As shown in Figure 2, the specific binding of [^{3}H]-FC to *Arabidopsis* membranes was a temperature-dependent process and was reversible, with a half-time of approximately 30 min at 20°C. Fusicoccin-binding in *Arabidopsis* is also heat-labile, since treatment of the membranes at 70°C for 10 min prior to the binding assay eliminates over 90% of the specific [^{3}H]-FC binding (data not shown). The results presented above are consistent with previous reports of [^{3}H]-FC binding to membrane fractions from spinach leaves (2), corn coleoptiles (4), *Vicia faba* leaf tissue (6), and oat roots (13).

To determine the saturation kinetics of [^{3}H]-FC binding, *Arabidopsis* membranes were incubated for 2 h with increasing concentrations of unlabeled fusicoccin (from 10^{-10} M to 10^{-6} M) in the presence of 10^{-9} M [^{3}H]-FC. As shown in Figure 3A, saturation is reached at about 10^{-6} M. A Scatchard plot of the data (Fig. 3B) indicates the presence of a single binding site with an apparent $K_d = 1.3 \times 10^{-8}$ M and N = 1.2 pmol/g fresh weight of *Arabidopsis* callus tissue. The apparent K_d reported here for *Arabidopsis* membranes is comparable to the lower affinity sites reported for spinach leaf membranes (2). From these binding data I was unable to detect the presence of high-affinity FC binding sites similar to those reported in some other plant tissues (2, 4, 6). It could be that this difference is due to my use of cultured tissue as a source of plant material, instead of intact leaves or roots. This is the first report of FC binding using cultured cells.

Now that FC activity and specific binding have been demonstrated in *Arabidopsis thaliana*, I will proceed with experiments aimed at isolating the gene coding for the presumed fusicoccin receptor using this plant.

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