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Partial Characterization of Fusicoccin Binding to Receptor Sites on Oat Root Membranes[1]

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RICHARD G. STOUT[2] AND ROBERT E. CLELAND
Botany Department, University of Washington, Seattle, Washington 98195

ABSTRACT

The possibility that fusicoccin (FC) binds to plasma membrane-associated ATPases of oat (cv. Victory) roots has been examined. Specific FC-binding *in vitro* is localized primarily on plasma membrane-enriched fractions. This FC-binding is greatly reduced by pretreatment of the membrane vesicles at temperatures above 45 C or with trypsin, and the same treatments cause the release of already bound FC. These results support the idea that the FC receptor is a protein located on the plasma membrane.

Both active ATPases and FC-binding proteins were solubilized using 1% Triton X-100. When this material was fractionated using gel chromatography, the ATPase activity could be separated from the FC-binding proteins. The identity of the FC-binding proteins is discussed with regard to the extensive evidence which supports the involvement of plasma membrane-ATPase H^+/K^+ pumps in FC-stimulated acidification and K^+ uptake.

FC[3], a phytotoxin isolated from the fungus *Fusicoccum amygdali* (2), rapidly stimulates net H^+ efflux and K^+ uptake in practically all higher plant tissues so far examined (17). FC has also been reported to stimulate plant cell enlargement (4, 19, 24), to cause rapid stomatal opening (25), and to promote seed germination (13). It has been suggested that the effects of FC on these important physiological processes may be a consequence of an FC-activated increase in H^+/K^+ exchange across the plasma membrane (16). Inasmuch as, in some instances, FC mimics the action of natural plant hormones (17), an understanding of the mechanism of action of FC may aid not only in the study of membrane transport in plants but also in studies on the mechanism of action of plant hormones.

The rapidity of the effects of FC on H^+ and K^+ transport (5, 21) and on hyperpolarization of the membrane potential (7, 18) suggests that FC may directly stimulate a H^+/K^+ exchange mechanism on the plasma membrane. A current hypothesis suggests that FC directly activates the PM-associated ATPases which may mediate the H^+/K^+ exchange. If so, FC might be expected to bind directly to PM-bound ATPases.

Dohrmann *et al.* (8) have shown that FC binding is localized on the PM-rich fractions of corn coleoptiles. It is not known, however, whether the FC is bound to the ATPases. Here we have examined the nature and location of FC binding in a different tissue, oat roots. Furthermore, we have solubilized both PM-ATPases and FC-binding proteins and have shown that they are not the same.

MATERIALS AND METHODS

Plant Material. Oat root tissue was selected as the material of study for three reasons. Oat roots rapidly excrete H^+ and take up K^+ in response to FC (6). Hodges *et al.* (10) have previously reported procedures for isolating PM-enriched membrane fractions from this tissue. Finally, relatively large quantities of oat root tissue can be easily obtained.

Seeds of *Avena sativa* L cv. Victory were germinated and grown hydroponically using the culture techniques of Hodges and Leonard (9). The oat seeds were grown in the dark for 4 to 5 days at about 25 C over a vigorously aerated solution of 1 mM $CaSO_4$. Whole roots (5-10 cm long) were excised and washed two to three times with cold distilled H_2O prior to homogenization.

Alternatively, seeds were sown on moist Vermiculite and grown in the dark at 28 C for 4 to 5 days. Intact seedlings were gently removed, and the roots were washed in distilled H_2O to remove the Vermiculite. The excised roots were washed three times with cold distilled H_2O before homogenization. Bacterial contamination of the oat roots grown in this manner was determined, and it was found that, after three washes in distilled H_2O, bacterial levels were reduced to about 10^4 bacteria/g fresh weight of tissue (Table I). No detectable differences in amount or activity of ATPases of the PM-enriched fractions were found between roots grown hydroponically and in Vermiculite.

Membrane Fraction Preparation. The procedures of Hodges and Leonard (9) were used to isolate membrane fractions, and all operations were carried out at 0 to 5 C. The roots were first chopped up with a razor blade and then homogenized using a mortar and pestle. Homogenization medium consisted of 0.25 M sucrose, 3 mM EDTA, 25 mM Mes-Tris (pH 7.2), and DTT (0.4 mg/ml), with about 4 ml homogenization medium/g fresh weight of tissue. The homogenate was strained through 4 layers of cheesecloth and centrifuged at 13,000*g* for 10 to 15 min. The 13,000*g* supernatant then was centrifuged at 80,000*g* for 30 min, and this crude membrane pellet was resuspended in 2 to 4 ml of resuspension medium (buffer A) consisting of 0.25 M sucrose, 1 mM $MgSO_4$, 0.5 mM EDTA, 10 mM Mes-Tris (pH 7.2), and DTT (0.4 mg/ml). The resuspended pellet was layered onto a discontinuous sucrose gradient consisting of 4 ml of 45% sucrose (w/w) and 6.4 ml each of 38, 34, 30, 25, and 20% sucrose in 1 mM $MgSO_4$, 1 mM Mes-Tris (pH 7.2), and DTT (0.4 mg/ml). This was centrifuged at 26,000 rpm in a Spinco SW 27 rotor for 3 h. The various membrane bands were removed from the multistep gradient using a pasteur pipette bent at the tip. To isolate only the plasma membrane-enriched vesicles, a one-step sucrose gradient consisting of 16 ml 34% sucrose and 20 ml 45% sucrose was used instead of the multistep gradient. The PM-enriched band was collected at

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[3] Abbreviations: FC, fusicoccin; DES, diethylstilbestrol; K^+-ATPase, K^+-stimulated adenosine triphosphatase; PM, plasma membrane.

Table I. *Effect of Rinsing Oat Roots Prior to Homogenization on Bacterial Contamination*

Roots were washed three times in sterile distilled H_2O and the rinse media were saved. A 1-ml aliquot of each of the three rinses and the homogenization medium were serially diluted up to 10^6-fold. A 0.1-ml aliquot from each dilution step was transferred to a Petri dish containing 2% potato dextrose agar (Difco) and incubated at 25 C. After 24 to 36 h, the number of colonies per plate were scored where possible. Assuming each colony arose from a single bacterium, the number of bacteria present in the original sample was calculated.

Sample	Bacterial Contamination in Experiment:			
	1	2	1	2
	total no.		*no./g fresh wt tissue*	
First rinse medium	1.6×10^9	6.8×10^9	4.6×10^7	1.7×10^8
Second rinse medium	1.2×10^7	5.7×10^7	3.4×10^5	1.4×10^6
Third rinse medium	3.3×10^6	6.5×10^6	9.4×10^4	1.6×10^5
Homogenization medium	3.5×10^5	1.1×10^6	1.0×10^4	2.8×10^4

the 34:45% interface.

The membrane fractions isolated on the sucrose gradients were immediately diluted 2- to 3-fold with distilled H_2O and centrifuged at 100,000*g* for 30 min. After centrifugation, the supernatant was removed and the membrane pellet was resuspended in 1 to 2 ml buffer A (0.5 to 2.0 mg membrane protein/ml) and stored at −15 C.

ATPase and Protein Assays. ATPase activity was assayed in a 0.5-ml volume containing 33 mM Mes-Tris (pH 6.5), 3 mM $MgSO_4$, 3 mM ATP (Tris salt, pH 6.5), 0.5 mM EDTA, and 50 mM KCl, when present. Reactions were initiated by the addition of 10 to 30 μg membrane protein. After 30 min at 37 C, the reaction was stopped with 1 ml of ice-cold 0.5% ammonium molybdate in 0.72 N H_2SO_4, and color was developed by addition of 50 μl of freshly prepared 10% ascorbic acid (w/v). Slight turbidity was clarified with 0.1 ml of 10% sodium lauryl sulfate (w/v). The *A* at 750 nm was determined after 20 min. K^+-stimulated ATPase (K^+-ATPase) activity is defined here as the difference in activities with and without 50 mM KCl.

Protein was assayed by the method of Lowry *et al.* (14) with BSA used as a standard. Possible interferences were corrected for by including in the standards the same components present in the buffers in which the protein samples were suspended.

[^{3}H]FC Binding. The PM-enriched fractions and the other microsomal fractions were assayed for specific [^{3}H]FC binding capacity by using the methods of Dohrmann *et al.* (8). Aliquots of the fractioned microsomal vesicles (0.2 to 0.5 mg membrane protein) were treated with ^{3}H-labeled dihydrofusicoccin (0.1 μM final concentration about 120,000 dpm; 0.55 Ci/mmol). Dihydrofusicoccin is known to have the same effect and activity on plant tissue as fusicoccin (1).

Duplicate samples were treated with [^{3}H]FC in the presence and absence of 0.1 mM unlabeled FC. Samples were brought to a 1 ml final volume using buffer A and incubated for 1 h at 25 C. Using 2-ml cellulose-nitrate tubes, the samples then were centrifuged at 100,000*g* for 30 min (Spinco Ti 50 rotor). The supernatants were removed and the inside of the tubes was rinsed very gently with 1 ml distilled H_2O. The pellet was resuspended in 1 ml 1% Triton X-100, using a sonicator (Bransonic 220, Bransonic Instrument Co., Shelton, Conn.) to disperse the membrane particles. Aliquots of the resuspended pellets were transferred to 10 ml Aquasol and radioactivity was counted using a Packard Tri-Carb liquid scintillation spectrometer.

The amount of specifically bound [^{3}H]FC was determined by subtracting the cpm associated with the membrane pellet in the presence of 1000-fold excess unlabeled FC (nonspecifically bound [^{3}H]FC) from the value obtained in the absence of unlabeled FC. [^{3}H]FC-binding is expressed in some experiments as the per cent of the total [^{3}H]FC added which is bound to the membrane material specifically.

Detergent Solubilization of Membrane Components. Triton X-100 was used to solubilize active ATPases and [^{3}H]FC-labeled components from membrane material. To a suspension of PM-enriched vesicles (2-5 mg membrane protein/ml) was added 20% Triton X-100 to yield a final concentration of 1 to 2% Triton (about 5-10 mg detergent/mg membrane protein). This mixture was sonicated from 2 to 5 min (Bransonic 220) and then stirred for 30 min at 5 C. The mixture was centrifuged at about 150,000*g* for 30 to 60 min, and the slightly amber supernatant was collected. Using this method, 40 to 50% of the detectable membrane protein was solubilized, including up to 80% of the K^+-ATPase activity and membrane-associated [^{3}H]FC (Table II). Triton-solubilized K^+-ATPase activity has two pH optima at 6.5 and 8.5. The K^+-ATPase at pH 8.5 is sensitive to sodium azide and the one at pH 6.5 is not (data not shown). This indicates that the solubilized pH 6.5 K^+-ATPase is from the plasma membrane, and the pH 8.5 K^+-ATPase is mitochondrial in origin.

Triton X-100, at the levels used here, interfered with both the ATPase and protein assays. Using the methods of Holloway (11), most of the detergent could be removed by incubating the solubilized material with moist BioBeads SM-2 (about 1 g BioBeads/50 mg Triton) for 1 h at 5 C with shaking.

Gel Filtration. A column (2 × 25 cm) of Bio-Gel P-6 (exclusion limit, about 6,000 mol wt) was used for separating [^{3}H]FC-labeled material from free [^{3}H]FC. A 0.5-ml sample was layered onto the column and eluted with a buffer consisting of 10 mM Mes-Tris (pH 7.2) and 0.1% Triton X-100. The flow rate was 20 to 30 ml/h at 5 C, and 1-ml fractions were collected.

A 1.6- × 45-cm column of Sepharose CL-6B 200 (exclusion limit, about 4×10^6 mol wt) was used for fractionation of detergent-solubilized proteins from PM-enriched vesicles. A 1-ml sample of solubilized membrane protein ([^{3}H]FC-labeled) was added to the column which was equilibrated with the elution buffer consisting of 50 mM sucrose, 1 mM $MgSO_4$, 0.5 mM EDTA, 10 mM Mes-Tris (pH 7.2), 1 mM DTT, and 0.05% Triton X-100. The flow rate was 4 to 5 ml/h at 5 C, and 2-ml fractions were collected.

Chemicals. Fusicoccin and radiolabeled dihydrofusicoccin [^{3}H]FC were a kind gift of Prof. E. Marré. ATP (Sigma; equine muscle, disodium salt) was converted to the Tris salt by Dowex 50

Table II. *Solubilization of Membrane Proteins, Including Active ATPases and Membrane-associated [^{3}H]FC, using Triton X-100*

Two 0.45-ml aliquots were taken from a sample of PM-enriched vesicles (pretreated with [^{3}H]FC) and to each aliquot was added 50 μl of 10% Triton X-100 to yield a final concentration of 1% Triton (about 10 mg Triton/mg membrane protein). One sample was exposed to 5 min of sonication (Bransonic 220), then both were incubated for 30 min at 5 C with gentle stirring. After centrifugation at 100,000*g* for 30 min, both the supernatants (solubilized material) and the resuspended pellets (nonsolubilized material) were assayed for protein, ATPase activity, and [^{3}H]FC.

Fraction	Sonication	Protein	K^+-ATPase	[^{3}H]FC
		mg/sample	*μmol Pi/ml·h*	*cpm/sample*
Vesicles		0.58	20.7	2,340
Supernatant	No	0.23 (45%)[a]	18.7 (65%)	1,200 (53%)
Pellet	No	0.28	10.1	950
Supernatant	Yes	0.32 (57%)	26.4 (92%)	1,650 (75%)
Pellet	Yes	0.23	2.5	475

[a] Per cent solubilized.

ion exchange chromatography. Trypsin (bovine pancreas, Code TL) was from Worthington Biochemical Corp., Freehold, N. J. Bio-Gel P-6 (100-200 wet mesh) and BioBeads SM-2 were purchased from Bio-Rad. The blue dextran 2000 was from Pharmacia. The Sepharose CL-6B (Pharmacia) and all other biochemicals were purchased from Sigma.

RESULTS

Preliminary experiments showed that oat root membrane preparations contain FC-binding sites. In corn coleoptiles, FC binding is associated primarily with the PM-rich fraction (8). In an attempt to localize FC-binding using oat root tissue, membrane fractions from oat roots were separated on a discontinuous sucrose density gradient (9), and each of the fractions was assayed for specific [^{3}H]FC-binding capacity and K^+-ATPase activity, *i.e.* the difference in activity with and without 50 mM KCl. This experiment was repeated three times. The absolute values of specific [^{3}H]FC binding and K^+-ATPase activity varied among the experiments due to differences in microsomal preparations. However, the relative values of [^{3}H]FC binding and K^+-ATPase activity among the five membrane fractions remained essentially the same. Specific [^{3}H]FC binding was localized primarily to those fractions (1.15 to 1.20 g/cc) previously identified at PM-enriched (9) but was present to some extent in all fractions (Fig. 1). It is possible that the specific [^{3}H]FC binding observed in the three lightest membrane fractions (1.08 to 1.15 g/cc) may result solely from PM contamination. This suggestion is supported by the presence in these fractions of K^+-ATPase (pH 6.5) activity, which may be considered as a marker for plant plasma membranes (9). Interestingly, the relative value of specific [^{3}H]FC binding in each of the five membrane fractions roughly parallels the amount of K^+-ATPase activity. Because the PM-enriched fraction displayed the greatest amount of specific [^{3}H]FC binding, this fraction was used for the remainder of the experiments.

In PM-rich fractions from corn coleoptiles, the pH optimum for [^{3}H]FC binding is about 5 to 6 (8). Specific [^{3}H]FC binding to oat root PM-enriched vesicles has a broad pH optimum from 5 to 7 (Fig. 2). Nonspecific [^{3}H]FC-binding does not seem to be affected by pH.

Table III shows that the number of available specific [^{3}H]FC-binding sites on the PM-enriched vesicles is significantly decreased by the treatment of the oat root tissue with unlabeled FC prior to homogenization. This provides evidence to support the presumption that the specific [^{3}H]FC-binding sites present on the PM-enriched vesicles are *in vivo* FC-receptor sites.

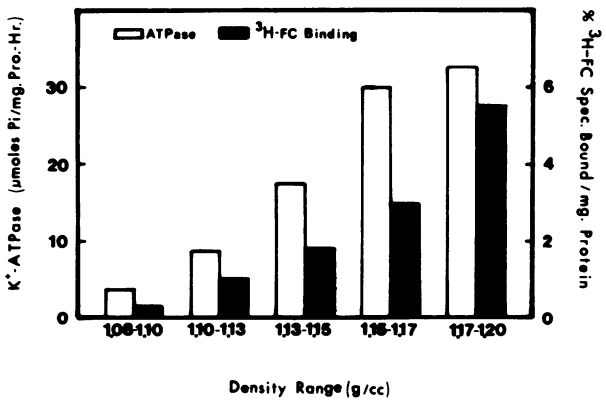

FIG. 1. Distribution of specific [^{3}H]FC binding and K^+-ATPase activity in five oat root membrane fractions separated on a discontinuous sucrose gradient. Each membrane fraction was removed from the gradient, diluted 2-fold with distilled H_2O, and repelleted at 100,000*g* for 30 min. Then each was resuspended in 1 ml of buffer A and 0.1-ml aliquots were assayed for [^{3}H]FC-binding capacity and ATPase activity.

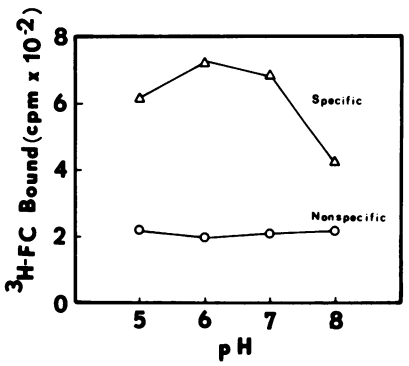

FIG. 2. The effect of pH on *in vitro* [^{3}H]FC binding to PM-rich vesicles from oat roots. Aliquots of the PM-rich material (about 0.2 mg membrane protein/sample) were incubated for 1 h at 25 C with 10 mM [^{3}H]FC (about 12,000 dpm) in a solution consisting of 50 mM sucrose, 0.2 mM $MgSO_4$, 0.1 mM EDTA, 0.2 mM DTT, and 50 mM Mes-Tris, at the various pH values shown, in the presence and absence of 0.1 mM FC. The membranes were pelleted and [^{3}H]FC binding determined.

Table III. *Effect of Pretreatment of Oat Roots with Unlabeled FC on the Specific [^{3}H]FC-binding Capacity of the PM-enriched Vesicles*

Two samples of excised oat root tissue (about 10 g fresh weight), harvested from the same batch of oat seedlings, were incubated for 1 h at 25 C in a 100-ml solution containing 0.2 mM $CaCl_2$ and 1 mM Mes-Tris, pH 7.2, with or without FC. Then PM-enriched fractions were isolated from each sample of oat roots, and the [^{3}H]FC-binding capacity of the PM-enriched vesicles was determined.

FC (10 μM) present *in Vivo*	[^{3}H]FC Binding	
	Nonspecific	Specific
	cpm/100 μg membrane protein	
−	142	382
+	163	164

There are reports in the literature concerning specific binding of plant hormones to non-plant material *in vitro* and possible hormone interaction with phospholipid bilayers (12). To investigate the possibility that FC may interact *in vitro* with protein or phospholipid moieties, the following experiment was performed. Labeled FC was incubated for 1 h at 25 C in a solution containing BSA, soybean phospholipids, or PM-enriched vesicles from oat roots (comparable levels of protein and phospholipids). Gel filtration using a Bio-Gel P-6 column was used to detect any bound [^{3}H]FC. Figure 3A shows that there was no detectable binding to BSA, and Figure 3b indicates that very little or no [^{3}H]FC binds to, or penetrates into, soybean phospholipid vesicles. Over 50% of the added [^{3}H]FC was associated with the PM-enriched vesicles (Fig. 3).

If the FC receptor is proteinaceous in nature, then it might be expected to be sensitive to heat denaturation and tryptic digestion. Figure 4 shows the effect of pretreatment of PM-enriched vesicles at various temperatures prior to [^{3}H]FC-binding and ATPase assays. Pretreatment at 45 and 60 C greatly reduced both the specific [^{3}H]FC-binding capacity and the K^+-ATPase activity. Trypsin treatment of the PM-enriched vesicles reduced the [^{3}H]FC-binding capacity and the K^+-ATPase activity by 75 and 85%, respectively (Fig. 5). The presence of trypsin inhibitor greatly reduced the effect of trypsin, ensuring that the results observed were not due to possible contaminants in the enzyme preparation.

To characterize further the nature of the FC receptor and to investigate the possible link between [^{3}H]FC binding and ATPase activity, PM-enriched vesicles were incubated with [^{3}H]FC, and

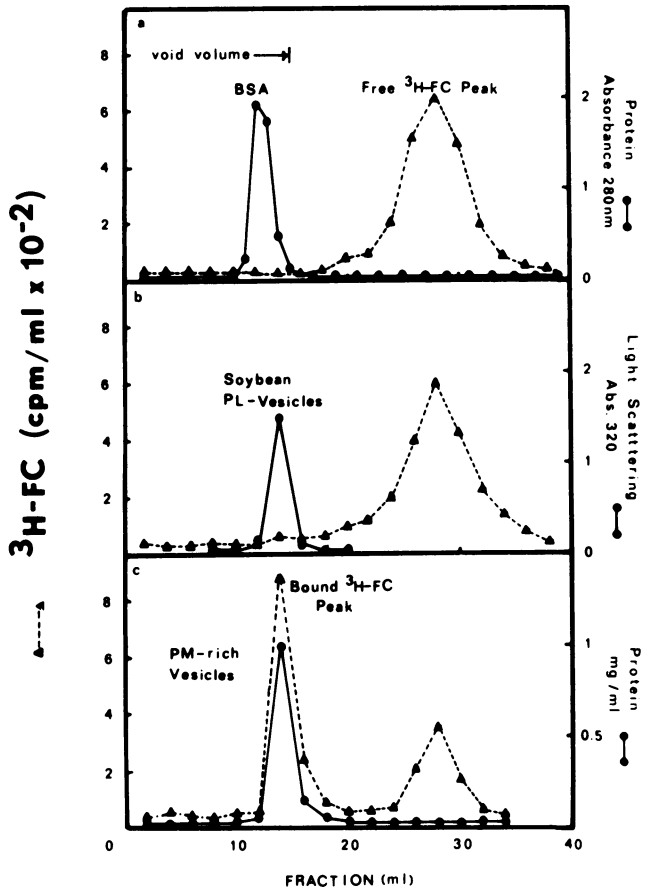

FIG. 3. A comparison at [^{3}H]FC binding to BSA, soybean phospholipids, and oat root PM-rich vesicles. The standard [^{3}H]FC-binding assay was performed and the assay mixtures were immediately passed through a Bio-Gel P-6 column to separate any bound [^{3}H]FC from the free [^{3}H]FC. ▲- - -▲, FC (cpm); ●——●, protein and light scattering.

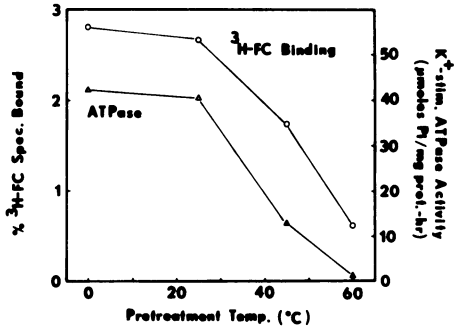

FIG. 4. The effect of pretreatment of PM-rich vesicles at various temperatures on specific [^{3}H]FC-binding capacity and K$^+$-ATPase activity. Four identical aliquots of PM-rich material were incubated for 30 min at 0, 25, 45, and 60 C. They then were returned to an ice bath, and [^{3}H]FC-binding and ATPase assays were performed.

then membrane-bound [^{3}H]FC and ATPases were solubilized. Both the nonionic detergent Triton X-100 and the bile salts cholate and deoxycholate were used. The results presented here will be limited to data obtained using Triton (Table II). We found that the effectiveness of the detergents was enhanced by 3 to 5 min of sonication just after adding the detergent or by the presence of 0.4 M ammonium sulfate (data not shown). Gel filtration of the Triton-solubilized material using Bio-Gel P-6 revealed that from 50 to 60% of the [^{3}H]FC was associated with the solubilized

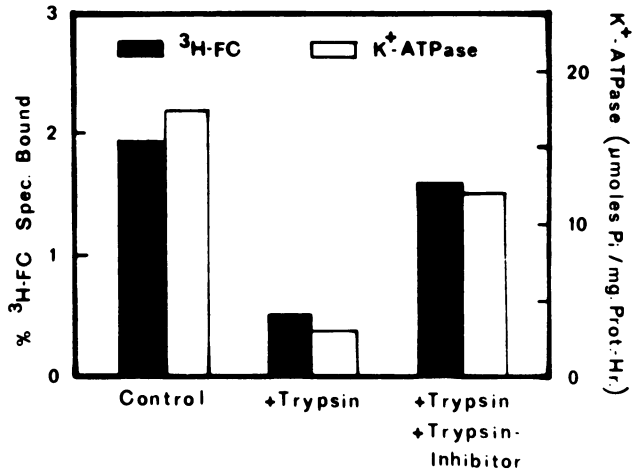

FIG. 5. Effect of trypsin on specific [^{3}H]FC binding and ATPase activity of PM-enriched vesicles. Three identical aliquots of PM-enriched vesicles were incubated for 1 h at 25 C with or without trypsin (1 mg trypsin/ml; specific activity, 60 units/mg protein) and with trypsin in the presence of trypsin inhibitor (1 mg trypsin inhibitor/ml, which reduced the specific activity of trypsin to about 10 units/mg protein). Then the samples were returned to an ice bath and immediately assayed for [^{3}H]FC-binding capacity and K$^+$-ATPase activity.

macromolecular membrane components (Fig. 6a). Thus, it was possible to solubilize not only active K$^+$-ATPases (Table II) but also [^{3}H]FC-labeled macromolecules (Fig. 6) from oat root PM-enriched vesicles.

Using solubilized [^{3}H]FC-labeled membrane components, we examined the effects of heat and trypsin treatments on the release of bound [^{3}H]FC (Fig. 6). Both 45 and 60 C treatments clearly promote the release of bound [^{3}H]FC. Essentially the same type of experiment was performed using trypsin in the presence and absence of trypsin inhibitor. As shown in Figure 7B, 30-min trypsin treatment caused the release of over 80% of the bound [^{3}H]FC. The inhibition of this release by trypsin inhibitor indicated that tryptic digestion, not some contaminant, was responsible for the observed results.

The Triton-solubilized proteins from PM-enriched vesicles retain the ability to bind [^{3}H]FC specifically (Fig. 8). When solubilized membrane proteins were incubated with [^{3}H]FC in the presence of excess unlabeled FC (which should block most or all of the FC-receptor sites), no [^{3}H]FC binding to the solubilized membrane proteins was detected (Fig. 8a). This shows that [^{3}H]FC does not bind nonspecifically to this material. However, when this experiment was repeated in the absence of unlabeled FC (potential FC-receptor sites exposed), about 2% of the added [^{3}H]FC (about 400 cpm) bound to some of the solubilized membrane proteins (Fig. 8b). Even when removed from the membrane environment by Triton solubilization, at least some of the FC-binding proteins retain the capacity to bind [^{3}H]FC specifically.

The results up to this point are consistent with the idea that FC binds to plasma membrane-associated proteins and possibly to ATPases. To determine whether FC-binding and ATPase activity coincide, we have fractionated detergent-solubilized proteins using gel chromatography. Preliminary results using Sephadex G-100 gel chromatography indicated that the apparent mol wt of the Triton-solubilized K$^+$-ATPase from oat root PM-enriched vesicles was greater than 100,000. Using a gel material with a relatively large exclusion limit (Sepharose CL-6B 200) to achieve better fractionation, a peak of K$^+$-ATPase activity could be separated from a peak of [^{3}H]FC-labeled proteins (Fig. 9). Blue dextran 2000 (maximum mol wt, 2×10^6) and BSA (mol wt 6.7×10^4) were used as mol wt markers. The mol wt range of the ATPase

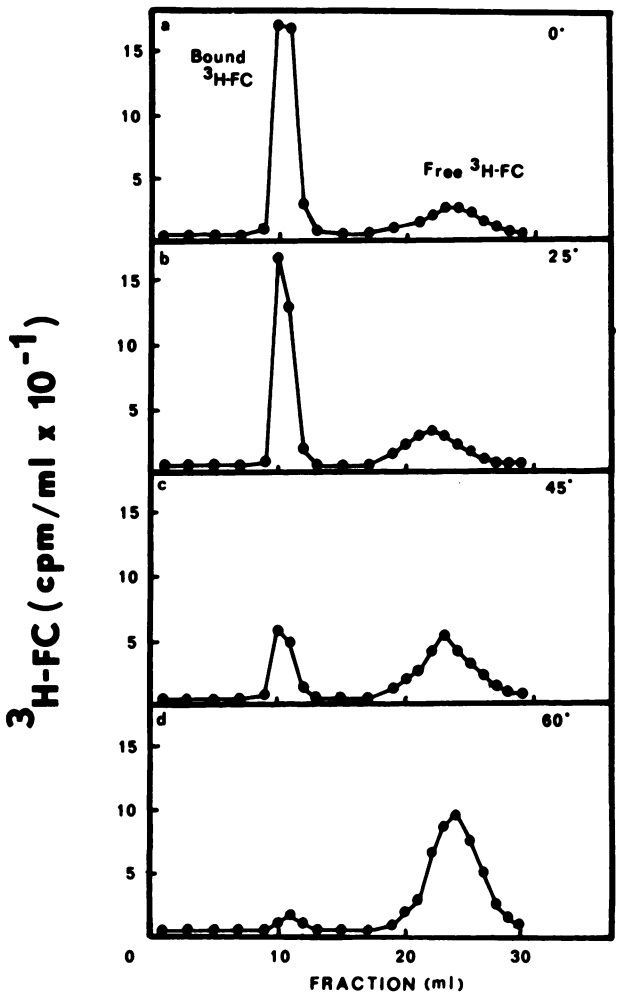

FIG. 6. Effect of treatment at various temperatures of solubilized [^{3}H]FC-labeled macromolecules on the release of the bound [^{3}H]FC. Four aliquots of Triton-solubilized material from PM-enriched vesicles (labeled with [^{3}H]FC) were incubated for 30 min at 0, 25, 45, and 60 C and then returned to an ice bath. Each sample was passed through a Bio-Gel P-6 column at 5 C to determine the amounts of bound and free [^{3}H]FC.

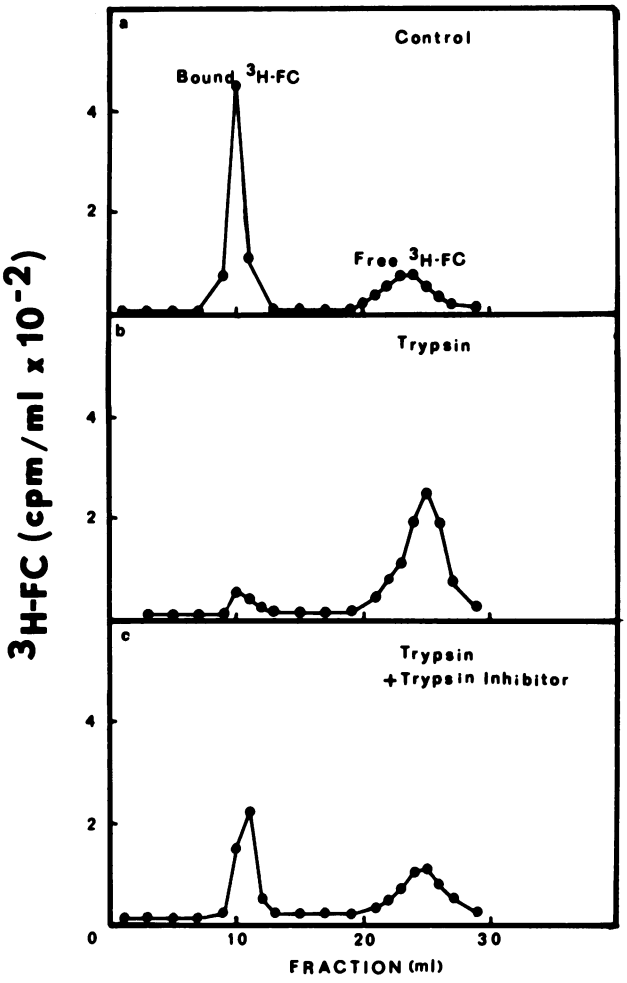

FIG. 7. Effect of trypsin treatment of solubilized [^{3}H]FC-labeled macromolecules on the release of the bound [^{3}H]FC. Three aliquots of Triton-solubilized material from PM-enriched vesicles (labeled with [^{3}H]FC) were incubated for 30 min at 25 C with or without trypsin and with trypsin in the presence of trypsin inhibitor (same amount and activity of trypsin and trypsin inhibitor as in Fig. 5), and the samples then were returned to an ice bath. Bound and free [^{3}H]FC were separated using gel filtration.

peak and the [^{3}H]FC-binding protein peak were roughly estimated to be 1.0 to 2.0 × 10^5 and 0.6 to 1.0 × 10^5, respectively. The ATPases isolated on the CL-6B 200 column are stimulated by 50 mM KCl sensitive to 0.1 mM dicyclohexylcarbodiimide and are insensitive to 0.1 mM DES (data not shown).

DISCUSSION

The results reported here support the idea that a protein associated with the plasma membrane may be the primary receptor for FC and that this protein is not an ATPase.

Previous reports concerning the thermolability of FC binding to microsomal preparations from corn coleoptiles (8) suggest that the FC-binding site is proteinaceous in nature. We have shown that the capacity of oat root membrane vesicles to bind [^{3}H]FC specifically is greatly reduced by protein-denaturing temperatures and by trypsin treatment. Using detergent-solubilized material containing [^{3}H]FC-labeled components, we have also shown that these same temperature and trypsin treatments promote the release of already bound [^{3}H]FC. These experiments, demonstrating the thermolability and trypsin sensitivity of specific [^{3}H]FC binding to oat root membrane material, provide further support for the idea that the FC receptor is a membrane protein.

FC binding is localized primarily on the membrane fractions of

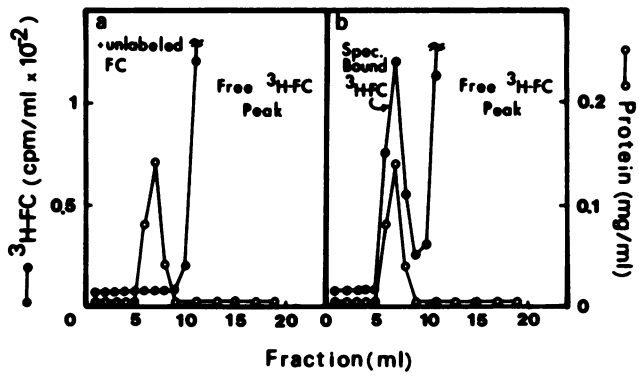

FIG. 8. [^{3}H]FC binding to solubilized proteins from oat root plasma membrane-rich vesicles. Two identical aliquots of Triton-solubilized material (0.5 mg protein/aliquot) were treated with [^{3}H]FC (0.1 μM, about 20,000 dpm) in the presence and absence of 0.1 mM unlabeled FC. Each sample was passed through a Bio-Gel P-6 column. Fractions 6 to 8 contained the solubilized proteins and fractions 10 to 25 contained the free [^{3}H]FC peak (not entirely shown).

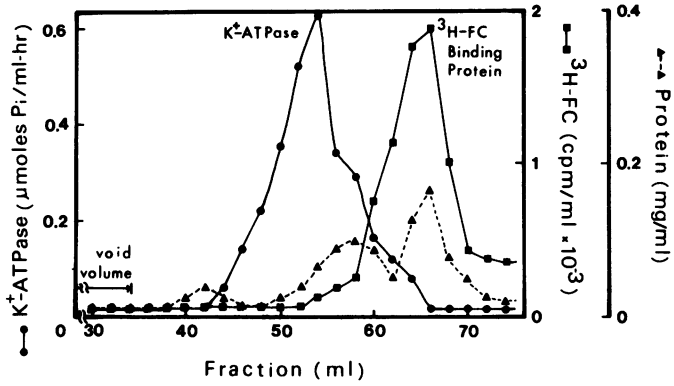

FIG. 9. Separation of solubilized K^+-ATPases and [^{3}H]FC-labeled proteins from oat root plasma membrane-rich vesicles using gel chromatoraphy. A 1-ml sample of Triton-solubilized, [^{3}H]FC-labeled proteins (about 6 mg total protein) was added to the Sepharose CL-6B column and 100 fractions were collected over a period of about 20 h. No protein or ATPase activity could be detected in the void volume. The free [^{3}H]FC peak was from fraction 76 through fraction 96 (not shown). ●——●, K^+-ATPase; ■——■, [^{3}H]FC; ▲- - -▲, protein.

oat roots which have previously been shown to be enriched in plasma membrane. This is in agreement with the result of Dohrmann *et al.* (8) and provides further evidence supporting the idea that FC acts at the cell surface. Since virtually all higher plant tissues tested to date excrete protons in response to FC, FC-binding sites must exist in all of these cells, presumably in the PM. But what are they doing there? Since FC is produced by the fungus *F. amygdali*, which attacks only a limited number of plants, it seems unlikely that the main function of this binding protein *in situ* is to act as an FC receptor. If it acts as a natural receptor, it must be a receptor for some different molecule.

What is the consequence of FC binding? It has been suggested that FC directly activates a PM-ATPase. This idea is supported by the fact that the effect of FC on the H^+/K^+ exchange is inhibited by treatments lowering the ATP levels in the tissue (20) and that inhibitors of ATPase activity *in vitro* also inhibit FC-stimulated H^+/K^+ exchange *in vivo* (21). There are also several reports of FC stimulation of ATPase activity *in vitro* (3, 15), although we have been unable to demonstrate any such effect. Using membrane fractions of oat root tissue, we have shown that the amount of specific binding of [^{3}H]FC roughly parallels the amount of K^+-ATPase activity in these fractions. We have also found that treatments which reduce the specific [^{3}H]FC-binding capacity in the PM-enriched vesicles also have similar effects on K^+-ATPase activity. The FC-receptor protein, which may be present on the cell surfaces of many different higher plant tissues, may be directly involved in ATP-driven proton and K^+ transport across the plasma membrane.

To investigate the possibility that FC binding and ATPase activity occur on the same proteins, we have detergent-solubilized membrane proteins, including both active K^+-ATPases and [^{3}H]FC-labeled proteins, from PM-rich fractions of oat roots and have fractioned them using gel chromatography. We have shown that the K^+-ATPases and FC receptors can be separated and, thus, are not identical. These results agree with those of Tognoli *et al.* (26), who used gel chromatography and disc electrophoresis to separate FC-receptor complexes and ATPases which they had solubilized from corn coleoptile microsomes using sodium perchlorate. Pesci *et al.* (23) have recently reported an approximate mol wt of 80,000 for the perchlorate-solubilized macromolecule-FC complex from the membrane fraction of corn coleoptiles. The striking similarities between our results and those of Marré and co-workers (24, 26) (even though we use different tissues and different solubilization and fractionation techniques) support the idea that the FC-receptor protein may be a common component of higher plant plasma membranes.

Although FC may stimulate H^+/K^+ exchange *in vivo* by activating an ATPase-coupled H^+/K^+ transport mechanism located on the plasma membrane, it appears that it does not do so by directly binding to an ATPase. What is the FC-binding protein?

One possibility is that it is a second component of a multiunit ATPase transport complex. Although it is not yet known whether the PM-ATPase is composed of more than one subunit, it seems likely that it is since most ATPase ion pumps from other organisms so far characterized are multiunit complexes (22). Marré has suggested that the FC-binder protein is the DES-sensitive part of the ATPase (17). This is based on the finding that DES interferes with the [^{3}H]FC binding to corn coleoptile microsomes (26) and the fact that the perchlorate-solubilized ATPase, which presumably lost the FC-binder portion, is no longer sensitive to DES (26). We doubt that there is a specific DES-sensitive site on the ATPase complex and would explain the DES inhibitory effects as due to perturbation of the membrane structure (27).

We feel that it is probable that the FC-binding protein may be associated in some way with an ATPase ion-transport complex. It is also possible, but unlikely, that the FC-binding protein may be part of a non-ATPase-mediated proton transport mechanism, may be an ATPase which is inactivated by removal from the membrane, or may even be a protein which is unrelated to the effects of FC *in vivo*.

We believe that the best way to resolve these questions is through reconstitution of a model membrane system containing both ATPases and FC-binding proteins.

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