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Functional reconstitution of a proton-translocating system responsive to fusicoccin

PATRIZIA ADUCCI*, ALESSANDRO BALLIO†‡, JEAN-PIERRE BLEIN§, MARIA ROSARIA FULLONE†, MICHEL ROSSIGNOL¶, AND RENÉ SCALLA§

*Dipartimento di Biologia, Università di Roma "Tor Vergata," 00173 Rome, Italy; †Dipartimento di Scienze Biochimiche, Università di Roma "La Sapienza," 00185 Rome, Italy; and §Laboratoire des Herbicides, and ¶Station de Physiophathologie Végétale, Institut National de la Recherche Agronomique B.V. 1540, 21034 Dijon cédex, France

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ABSTRACT **Crude fusicoccin binding proteins and a partially purified plasma membrane H^+-transporting ATPase (EC 3.6.1.34), both solubilized from maize tissues, were simultaneously inserted into liposomes by the freeze–thaw method. ATP-driven intravesicular acidification in the proteoliposomes, measured by the fluorescence quenching of the dye 9-amino-6-chloro-2-methoxyacridine, markedly increased upon addition of fusicoccin to the reconstituted system. This effect could not be observed when binding sites and ATPase preparations were separately reconstituted into the proteoliposomes, thus demonstrating that fusicoccin binding to its receptor is a prerequisite for ATPase stimulation.**

Fusicoccin (FC) (1, 2) is a fungal metabolite that affects several physiological processes normally controlled by plant hormones. Stomatal movement, cell enlargement of various tissues, and germination of dormant seeds are stimulated by concentrations as low as 1–10 μM in a large number of higher plants (3). These effects are tissue-nonspecific, are evoked very rapidly, and are not suppressed by transcription or translation inhibitors (3). Probably FC can short-circuit at least part of the transduction chain of the hormones; thus, the elucidation of its mechanism of action might shed some light on that of plant hormones.

Evidence has been presented that the first step in the mode of action of FC is its recognition by high-affinity and specificity-binding sites (4). In fact, these sites are saturated by a ligand concentration close to that giving an optimal response *in vivo* (5–8) and bind FC derivatives and analogs in a manner that parallels their biological activities (9). The nature of the step following FC binding—namely, the transduction mechanism of the signal to the cell machinery—is quite obscure. Circumstantial evidence suggests that the target molecule in higher plants is the plasma membrane H^+-transporting ATPase (EC 3.6.1.34) (3), but attempts to demonstrate a substantial *in vitro* FC stimulation of this enzyme have yielded contradictory results (10–13).

Recently, a reproducible and specific stimulation of the H^+-ATPase activity in radish microsomal vesicles incubated with FC has been claimed (14, 15). Like FC binding to microsomal preparations of maize (5, 7, 8) and of other plants (16, 17), this stimulation exhibits saturation kinetics, reaching saturation at a 0.1 μM concentration. These results support the existence at the plasma membrane level of a functional relationship between the FC-binding protein and the H^+-ATPase, but experimental support for this hypothesis is still lacking.

In this paper we show that FC can stimulate H^+-pumping in a system obtained by incorporating into liposomes a partially purified plasma membrane H^+-ATPase from maize roots or shoots together with a crude preparation of FC-binding sites from maize shoots.

MATERIALS AND METHODS

ATPase. The membrane-bound enzyme was obtained from maize seedlings by a recently described procedure (18) that affords a preparation insensitive to 100 mM nitrate, 1 mM molybdate, or 5 mM azide but 90% inhibited by 100 μM vanadate and 100% dependent on magnesium. Its specific activity is in the range of 2–7 μmol of inorganic phosphate hydrolyzed per min per mg of protein at 38°C. The enzyme activity was solubilized with lysolecithin and precipitated with ammonium sulfate as reported (18).

FC-Binding Sites. An acetone-dried microsomal fraction was prepared from shoots of 7-day-old etiolated hydroponically grown maize (*Zea mays* L.) seedlings by using the procedure followed for spinach leaves (19, 20), with the only exception being that the grinding medium also contained 0.1 mM phenylmethylsulfonyl fluoride and 0.1 mM *p*-hydroxymercuribenzoate. Solubilization of this material was achieved by the procedure used for FC-binding proteins of spinach leaves (21).

Reconstitution Experiments. Liposomes were prepared by sonicating in a bath sonicator (Laboratories Supplies, Hicksville, NY) a mixture of 25 mg of soybean phosphatidylcholine (type II, Sigma) and 25 mg of sheep brain phosphatidylethanolamine (type II, Sigma) suspended in 1 ml of 50 mM Tris/Mes buffer (pH 6.5) containing 100 mM KCl. Reconstitution was achieved by the freeze–thaw procedure (22). Briefly, a mixture of ATPase (20 μl containing 20 μg of protein with a specific activity of 3.1 μmol of inorganic phosphate hydrolyzed per min per mg of protein at 38°C and pH 6.5) preincubated for 5 min at room temperature with lysolecithin (20 μg in 5 μl), liposomes (90 μl containing 4.5 mg of phospholipids), and 1 M KCl (5 μl) was kept for 5 min at −80°C and then thawed at room temperature. The freeze–thaw treatment was repeated after addition of FC-binding sites (10 μl containing 40 μg of protein). When required, proteoliposomes were preloaded with FC by adding the substance to the ATPase/lysolecithin mixture immediately before the first freeze–thaw step.

Proton Transport Assay. ATP-dependent H^+-translocation was measured by the initial rate of fluorescence quenching of the dye 9-amino-6-chloro-2-methoxyacridine (23). The assay medium (2 ml) contained 2 μM dye, 10 mM Tris/Mes buffer (pH 6.5), 100 mM KCl, 5 mM $MgSO_4$, and 130 μl of proteoliposomes prepared as described above. Fluorescence quenching of the dye was monitored in a thermostated cell at 26°C with a Jobin–Yvon JY3D spectrofluorimeter with excitation and emission wavelengths of 430 and 500 nm, respec-

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Abbreviation: FC, fusicoccin.

‡To whom reprint requests should be addressed.

tively. During the measurements the samples were continuously stirred. After a short temperature equilibration, H^+-pumping was initiated by addition of 3 mM ATP. The H^+ gradient was discharged by addition of ionophores (0.5 μM valinomycin and 5 μM carbonyl cyanide *m*-chlorophenylhydrazone). The initial rate of quenching was calculated as quenching percentage of the fluorescence recovered after the addition of ionophores at the end of each experiment.

Chemicals. FC was prepared as described by Ballio *et al.* (24). The dye 9-amino-6-chloro-2-methoxyacridine was a gift of A. Goffeau (University of Louvain, Louvain-la-Neuve, Belgium). All other products were of analytical grade.

RESULTS AND DISCUSSION

The choice of maize tissues for the present study was dictated by the experience accumulated during previous investigations. In fact, binding of FC to plant membranes has been demonstrated for the first time in the microsomal fraction of maize coleoptiles (5), which also has been used for the kinetic study of the binding reaction and for a preliminary biochemical characterization of the binding proteins (6–8, 25, 26). The FC-binding sites solubilized from maize roots or shoots have also been reconstituted into phospholipids to yield proteoliposomes that display binding properties close to those of microsomal preparations (unpublished results). In a recent report (18), the preparation from a membrane fraction of maize roots of a H^+-ATPase highly sensitive to vanadate and almost completely free of phosphohydrolase activities originating from membranes other than plasmalemma, its solubilization, and its subsequent incorporation into liposomes have been described.

The insertion into liposomes of both FC-binding sites and H^+-ATPase-enriched preparations has been attempted by several methods. Good results have been obtained with the freeze–thaw procedure (22), which therefore has been used in most experiments. Reproducible data have also been produced with proteoliposomes prepared by the octyl glucoside dilution method (18, 27).

Fig. 1 shows that the H^+-translocating activity of the reconstituted ATPase, assayed by the quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence upon addition of ATP, is well preserved by proteoliposomes that also have incorporated a crude preparation of FC-binding sites (curve a). This activity was significantly enhanced when the proteoliposomes were briefly incubated with FC before ATP addition (curve b) and was increased further when some FC was incorporated inside the proteoliposomes (curve c).

Qualitatively similar results were obtained when the ATPase preparation used for reconstitution was solubilized from shoots rather than roots of maize or when the proteoliposomes were prepared by the octyl glucoside procedure. Irrespective of the reconstitution method, the H^+-transport rate or the phosphohydrolase activity of the ATPase preparation was not affected by FC when the binding sites were omitted. Moreover, H^+-pumping was not observed when the proteoliposomes were reconstituted with the crude FC-binding proteins only—namely, without the H^+-ATPase-enriched preparation. The reproducibility of the data shown in the figure is shown by the values included in Table 1. These were derived from five separate experiments carried out with the same batches of enzyme and FC-binding protein preparations. With different batches, a change in H^+-pumping extent was sometimes noticed, probably as a consequence of differences in the specific activities of the preparations, but the modulation induced by FC was consistently observed.

This investigation shows that an artificial system obtained by incorporating into liposomes a mixture of preparations enriched in plasma membrane H^+-ATPase and FC-binding protein, both solubilized from maize tissues, can duplicate

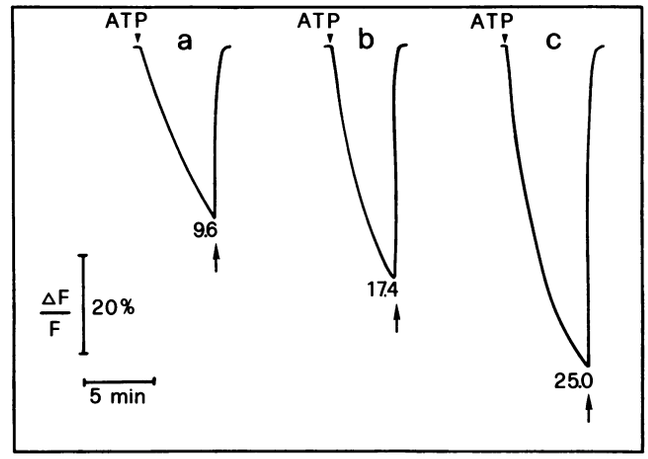

FIG. 1. Effect of FC on ATP-driven H^+-transport in reconstituted liposomes. Acidification of proteoliposomes is measured by fluorescence (F) quenching of the dye 9-amino-6-chloro-2-methoxyacridine. The initial rate of quenching, expressed as the percentage of total fluorescence per minute, is indicated on the curves. Acidification is initiated by adding ATP (arrowheads), and the consequent gradient is discharged by addition of 0.5 μM valinomycin and 5 μM carbonyl cyanide *m*-chlorophenylhydrazone (arrows). Curves: a, proteoliposomes containing ATPase and FC-binding sites (control); b, same proteoliposomes but briefly incubated with 1 μM FC before ATP addition; c, same proteoliposomes but preloaded with 1 μM FC.

the stimulation of H^+-pumping observed when native vesicles of radish are incubated with FC. Since the ATP-dependent H^+-transport rate of the H^+-ATPase reconstituted without FC-binding sites is unchanged upon addition of FC, an effect of this substance on phospholipid membrane with consequent alteration of the percentage of sealed vesicles or of the amount of reconstituted ATPase can be ruled out. Some years ago it was observed that FC does not influence passive transport or electric conductance in natural or artificial membranes (28). The clear separation of H^+-pumping activity, via the ATPase, from its modulation by FC, associated with the FC-binding protein, indicates that the interaction of FC with its specific binding sites is an indispensable prerequisite for its activating effect on the plasma membrane H^+-ATPase. At the moment it is impossible to decide if the signal decoded by FC-binding sites is directly transmitted to the ATPase or reaches the target molecule through a more or less complicated transduction chain. In fact, both FC-binding sites and H^+-ATPase used in the experiments reported in this paper are far from being pure proteins. It has been shown that they can be separated from each other (17, 26), and since they are located on opposite sides of the plasma membrane, it will be of interest to find out how communication between them is achieved in the plant cell.

The experimental approach used in this paper—namely, the simultaneous incorporation into liposomes of a plant growth regulator-binding site and a H^+-ATPase—might represent a tool for investigating the mode of action of some

Table 1. Effect of FC on H^+-pumping of proteoliposomes reconstituted with H^+-ATPase and FC-binding sites

FC additions	H^+-pumping rate,* mean ± SEM	% stimulation
None	10.50 ± 0.88	—
1 μM		
Outside only	15.90 ± 1.50	+51
Outside and inside	18.40 ± 2.42	+75

*% fluorescence quenching per min.

plant hormones. The technique of reconstitution of specific solubilized membrane proteins has been little used in plant work: to our knowledge the only report published so far concerns the generation of an auxin–ATP-dependent electrochemical response on reconstitution of a solubilized auxin receptor preparation, which also contains ATPase activity, into a bilayer lipid membrane (29).

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