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Patrizia Aducci, Alessandro Ballio, Jean-Pierre Blein, Maria Rosaria Fullone, Michel Rossignol, René Scalla

Reconstitution of a proton-translocating system responsive to fusicoccin stimulation

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Biochimica. – *Reconstitution of a proton-translocating system responsive to fusicoccin stimulation.* Nota di PATRIZIA ADUCCI [1], ALESSANDRO BALLIO [2], JEAN-PIERRE BLEIN [3], MARIA ROSARIA FULLONE [1], MICHEL ROSSIGNOL [4] e RENÈ SCALLA [3], presentata [*] dal Socio G.B. MARINI-BETTOLO.

ABSTRACT. – Proteoliposomes containing both fusicoccin-binding sites and plasmalemma ATPase, separately solubilised from maize tissues, have been obtained by a freeze-thaw procedure. The proton gradient generated by ATP is markedly stimulated on addition of fusicoccin; if the proteoliposomes contain only one of the two protein preparations the stimulation is not observed. The results show that the binding of fusicoccin to its receptors is a prerequisite for ATPase stimulation.

KEY WORDS: Fusicoccin; Proteoliposomes; Receptors.

RIASSUNTO. – *Ricostituzione di un sistema di traslocazione protonica stimolabile da fusicoccina.* Mediante un metodo di congelamento e scongelamento rapido sono stati ottenuti proteoliposomi in cui erano simultaneamente inseriti siti leganti fusicoccina (FC) e ATPasi di plasmalemma, solubilizzati separatamente da tessuti di mais. Dopo aggiunta di FC si osserva un forte incremento del gradiente protonico generato da ATP, misurato mediante una sonda di fluorescenza. L'effetto non è osservabile se i siti leganti o l'ATPasi sono ricostituiti separatamente nei liposomi. Questi risultati rappresentano perciò la dimostrazione che il legame della FC ai recettori è premessa necessaria per la stimolazione della ATPasi.

INTRODUCTION

Fusicoccin (FC) (1,2) is a fungal metabolite with remarkable plant growth regulation properties, very likely resulting from the stimulation of the proton-translocating pump associated with the plasma membrane (3). Evidence has been presented that the first step in the mode of action of this substance is its interaction with high affinity and specificity binding sites (4). These sites are saturated by a ligand concentration close to that giving an optimal response *in vivo* (5-8), and bind

(1) Dipartimento di Biologia, Università di Roma «Tor Vergata», 00173 Roma, Italy.
(2) Dipartimento di Scienze Biochimiche, Università di Roma «La Sapienza», 00185 Roma, Italy.
(3) Laboratoire des Herbicides, I.N.R.A., B.V. 1540, 21034 Dijon-cédex, France.
(4) Station des Physiopatologie Végétale, I.N.R.A.., B.V. 1540, 21034 Dijon-cédex, France.
(*) Nella seduta del 23 aprile 1988.

FC derivatives and analogues in a measure which parallels their biological activities (9). The nature of the steps following FC binding, namely the transduction mechanism of the signal to the cell machinery, is quite obscure. The target molecule in higher plants is very likely represented by the plasma membrane ATPase (3), but attempts to demonstrate that FC directly stimulates this enzyme *in vitro* have yielded contradictory results (10-13).

Recently a reproducible and specific stimulation of the ATPase activity has been observed in radish microsomal vesicles incubated with FC (14,15). Similarly to the binding to microsomal preparations of maize (5,7,8) and other tissues (16,17), this stimulation exhibits saturation kinetics; both processes reach saturation at similar concentrations (ca 0.1 μM). These results support the existence at the plasma membrane level of a functional relationship between the FC binding protein and the proton translocating ATPase (3), but experimental support for this hypothesis is still lacking.

New information about the relationship between the two proteins has been sought by reconstituting into proteliposomes the plasma membrane ATPase of maize roots or shoots together with the FC binding sites from maize shoots, and observing the influence of added FC on proton pumping.

Materials and methods

ATPase. Membrane fractions were prepared from 7 days-old etiolated hydroponically-grown seedlings of maize (*Zea mays* L.) by a modification of a previously described procedure (18,19). After grinding, the homogenates of roots or shoots were centrifuged at 16,000 *g* for 30 min. and the supernatants pelletted at 57,000 *g* for 30 min. Pellets were resuspended and centrifuged through a 30% sucrose cushion at 90,000 *g* for 90 min to afford the microsomal fraction. After washing, first with 0.25% Triton X-100 and 0.5 M KBr and then with 0.5 M KBr, this fraction had an ATPase activity (specific activity in the range 2-7 μmol Pi hydrolysed min^{-1} mg^{-1}) very sensitive to vadanate (I = 13 μM). The ATPase was solubilised with lysolecithin at a detergent to protein ratio of 5 and finally made free of lysolecithin by ammonium sulphate precipitation.

FC binding sites. An acetone powder was prepared from shoots of 7 days old etiolated hydroponically-grown maize seedlings according to the procedure followed for spinach leaves (20), with the only exception that the grinding medium also contained 0.1 mM phenylmethysulphonyl fluoride and 0.1 mM p-hydroxymercuribenzoate. Solubilisation of the binding proteins was achieved by the procedure described for microsomal preparations of spinach leaves (20,21).

Reconstitution of proteoliposomes. Liposomes were prepared by sonicating in a bath sonicator (Laboratories Supplies Company, Inc.) a mixture of 25 mg soybean phosphatidylcholine (type II, Sigma) and 25 mg sheep brain phosphatidylethanolamine (type II, Sigma) suspended in 1 ml of 50 mM Tris-Mes buffer pH 6.5. Reconstitu-

tion was achieved by the freeze-thaw procedure method (22). Briefly, a mixture of ATPase (20 μl containing 20 μg protein with specific activity 3.1 μmol inorganic phosphate hydrolysed min^{-1} mg^{-1} 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 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 protein). When required, proteoliposomes were preloaded with FC by adding the substance to the ATPase-lysolecithin mixture immediately before the first freeze-thaw step.

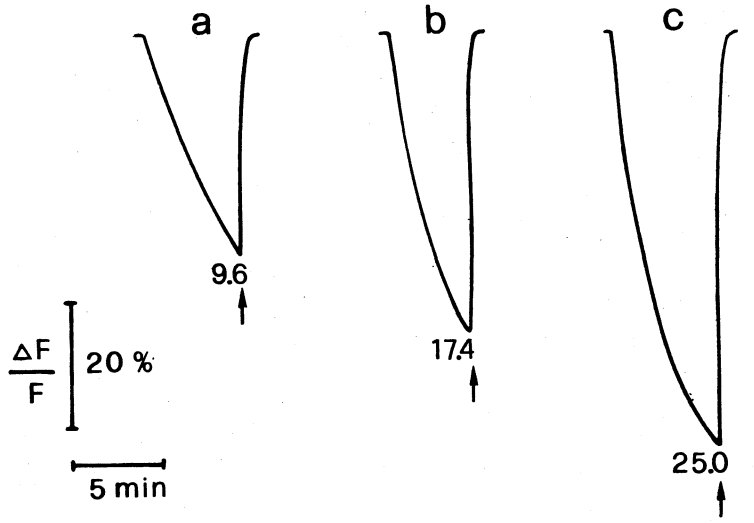

Fig. 1. - Effect of FC on ATP-driven proton transport in reconstituted vesicles. *a*. Proteoliposomes containing ATPase and FC-binding sites (control); *b*. Same, shortly incubated with 1 μM FC before ATP addition. *c*. Same, but proteoliposomes preloaded with 1 μM FC. Arrows indicate the addition of 0.5 μM valinomycin plus 5 μM carbonyl cyanide m-chlorophenylhydrazone. The initial rate of quenching, expressed as percentage of total fluorescence per minute, is indicated on the curves.

Proton transport assay. Mg:ATP-dependent acidification inside the proteoliposomes 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. The fluorescence decrease with time 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, respectively. During the measurements, the samples were continuously stirred. H^+ translocation was initiated by addition of 3 mM ATP after short temperature

equilibration. The H^+ gradient was discharged by addition of the ionophores 0.5 μM valinomycin and 5 μM carbonyl cyanide m-chlorophenyl-hydrazone. The initial rate of quenching was expressed as percentage of total fluorescence per minute.

Chemicals. FC was prepared according to Ballio et al. (24). The dye 9-amino-6-chloro-2-methoxyacridine was a kind gift of Prof. A. Goffeau, University of Louvain, Louvain-la-Neuve, Belgium. All other chemicals were of the best commercial grade.

Results and discussion

The choice of maize tissue for the present study was dictated by the experience accumulated during previous investigations. Binding of FC to plant membranes was demonstrated for the first time in microsomal preparations of maize coleoptiles (5), which consequently were chosen for the kinetic study of the binding reaction and for a preliminary biochemical characterization of the binding sites (6-8,25,26). A solubilised preparation of maize plasma membrane ATPase was obtained by following a procedure previously developed for ther *Acer pseudoplatanus* enzyme (19). It was highly sensitive to vanadate inhibition. Insertion into liposomes of FC binding sites and plasma membrane ATPase was attempted by several methods. The freeze-thaw procedure gave very good results and was in fact utilized in most experiments; the same results were obtained when the experiments were performed with proteoliposomes reconstituted by the octyl glucoside dilution procedure (27). As shown in the figure, the ATPase reconstituted together with the FC binding sites retains the capacity of transporting protons upon addition of ATP (curve *a*). When the mixture is shortly incubated with FC before addition of ATP, a significant enhancement of proton uptake takes place (curve *b*). This is more pronounced if the proteoliposomes are also preloaded with some FC (curve *c*). Whatever the reconstitution method, the proton transport rate is not influenced by FC if binding sites are omitted. Moreover, no ATP-dependent proton transport takes place when the reconstitution is performed with binding sites alone. The FC stimulation of proton transport is also observed when the system is reconstituted with the ATPase solubilised from shoots rather than roots of maize.

This investigation shows that an artificial system, obtained by inserting into liposomes a mixture of preparations of ATPase and FC binding sites solubilised from maize tissues, can duplicate the stimulation of proton pumping observed when native vesicles of radish are incubated with FC. Thus, it is clear that the activation *in vitro* of the plasma membrane proton ATPase by FC has an absolute requirement for FC binding proteins. It is worthwhile noting that the methodological approach used for the present work, namely the analysis of a protein-protein interaction by resolution of its components, followed by their reconstitution into a functional system, can be further exploited for the study of the mode of action of FC. In fact, it will guide experiments designed to establish if the signal consequent to the interac-

tion of the effector molecule with its binding sites is directly transduced to the AT-Pase, or if it enters into a more complicated pathway. Perhaps the same approach might also help in unravelling aspects of the molecular mode of action of some plant hormones. In this respect, the demonstration that an auxin-ATP-dependent electrochemical response is generated on reconstitution of a solubilised auxin receptor preparation, which also contains ATPase activity, into a bilayer lipid membrane has particular relevance (28).

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