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FUSICOCCIN STIMULATES THE H^+-ATPase OF PLASMALEMMA IN ISOLATED MEMBRANE VESICLES FROM RADISH

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The effect of fusicoccin on the plasmalemma H^+-ATPase has been investigated in a membrane fraction from 24 h old radish seedlings, in which Mg:ATP-dependent H^+-transport is mediated only by the plasmalemma H^+-ATPase. Fusicoccin stimulated the plasmalemma H^+-ATPase - i.e. Mg:ATP-dependent intravesicular acidification, hyperpolaryzation of $\Delta\Psi$ and ATPase activity -, when these activities were measured at the physiologically relevant pHs of 7.3 to 7.6. No effect of FC on the plasmalemma H^+-ATPase was evident at pH 6.6.

The H^+-ATPase of the plasmalemma (PM) of higher plants seems to play a key role in many physiological processes, such as intracellular pH regulation, active transport of ions and solutes, growth by cell enlargement, etc (1,2).

Evidence from "in vivo" work led to the hypothesis that the activity of this H^+-ATPase is directly stimulated by fusicoccin (FC), the fungal toxin produced by Fusicoccum amygdali (3). However a clear-cut demonstration of a stimulation by FC of PM H^+-ATPase activity is, up to now, lacking.

A stimulating effect of FC on ATPase activity in microsomal fractions has been first reported by Beffagna et al. (4) and subsequently confirmed by other Authors (5,6), but the observed changes were relatively small and in many cases no stimulation was detected (7,8). So the possibility that FC only indirectly stimulates electrogenic proton extrusion, for example through an acidification of the cytoplasm (9,10), is still open.

In this paper we show that, in microsomal vesicles from radish seedlings, FC strongly stimulates the H^+-transport activity and, to a lesser extent, the

Abbreviations: PM, plasmalemma; FC, fusicoccin; Bis-Tris, 2-bis(2-hydroxyethyl) amino-2-(hydroxymethyl)-1,3-propane-diol; Hepes, N-2-hydroxyethylpiperazine -N'-2-ethanesulfonic acid; AO, Acridine orange; MES, 2'(N-morpholino)--ethanesulfonic acid; OX VI, bis(3-propyl-5-oxoisoxazol-4-yl)pentamethineoxonol; $\Delta\Psi$, membrane potential difference.

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phosphohydrolytic activity of the PM H^+-ATPase measured at the physiologically relevant pHs.

MATERIALS AND METHODS

Microsomal vesicles from 24 h old radish seedlings (Raphanus sativus L. cv. Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) prepared as previously described (11) were resuspended in 0.25 M sucrose, 0.2% BSA, 0.5 mM ß-mercaptoethanol, 0.25 mM $CaSO_4$, 4 mM $MgSO_4$, 1 mM Bis-Tris-Hepes pH 7.0, at 2-3 mg membrane protein ml^{-1}.

Pretreatment with FC: Aliquots of membrane suspension (1 ml) additioned with 20 µl of 0.25 mM FC (in 0.15% ethanol) or of 0.15% ethanol, were incubated for 15 min at 25°C. Membrane suspensions were then maintained in an ice-bath for up to 4 h without significant changes in H^+-transport or ATPase activity.

Intravesicular acidification: Mg:ATP-dependent intravesicular acidification was monitored as the initial rate of decrease of AO absorbance (ΔA 492-550) utilizing a dual wavelength Sigma ZWS II spectrophotometer (11).
Membrane vesicles (25 ul) preincubated ±FC were incubated in 1.5 ml of 150 mM KBr, 5 mM $MgSO_4$, 0.2 mM EGTA, 3 µM AO, 40 mM Tris-Cl (pH 7.3 to 7.5) or MES-Tris (pH 6.6) plus 5 µM FC (FC pretreated samples) or the corresponding ethanol (controls).
The samples were incubated for 25-30 min at 25°C in the dark to equilibrate the intravesicular pH with the pH of the assay medium; then the reaction was started by addition of 15 µl of 0.3 M Na_2 ATP (pH 6.6 or 7.4 with Tris).

$\Delta\Psi$: Mg:ATP-dependent hyperpolaryzation of $\Delta\Psi$ was monitored as the increase of OX VI absorbance (ΔA 630-608).
Membrane vesicles (10 µl) pretreated ±FC were incubated in 1.5 ml of 75 mM K_2SO_4, 40 mM Tris-SO_4 pH 7.4, 5 mM $MgSO_4$, 0.2 mM EGTA, 3 µM OX VI, 5 mM $(NH_4)_2SO_4$ ±5 µM FC for 5 min at 25°C in the dark. Reaction was initiated by addition of 15 µl of 0.3 M Na_2 ATP (pH 7.4 with Tris).

ATPase: Membrane vesicles (20 µl) pretreated ±FC were incubated in 1 ml of 100 mM KNO_3, 5 mM $MgSO_4$, 40 mM Tris-Cl (pH 7.4 to 7.5) or MES-Tris (pH 6.6), 5 mM NaN_3, 0.2 mM EGTA, 100 µM ammonium molybdate, 5 mM $(NH_4)_2SO_4$, 3 mM ATP ±5 µM FC for 1 h at 25°C.
The released Pi was assayed according to Cross et al. (12).

Protein: Membrane protein were determined as previously described (13).

Experiments were run at least twice with three or more replicates. Variability of replicates within each experiment did not exceed ±5% (Δ pH and $\Delta\Psi$) or ±3% (ATPase) of the reported values.

RESULTS AND DISCUSSION

We previously reported that in microsomal vesicles obtained from 24 h old radish seedlings Mg:ATP-dependent electrogenic H^+-transport is mediated only by the vanadate-sensitive H^+-ATPase of plasmalemma (11).

We investigated the effect of FC on Mg:ATP-dependent intravesicular acidification and on ATPase activity in this microsomal fraction.

Table 1. Effect of FC on Mg:ATP-dependent intravesicular acidification and ATPase activity

pH of the medium	intravesicular acidification [a]		ATPase [b]	
	-	FC	-	FC
6.60	0.230	0.240	9.03	9.41
7.50	0.017	0.038	3.33	4.83

a) - $\Delta A_{(492-550)} \times min^{-1} \times mg^{-1}$ protein x ml.

b) - μmol $P_i \times h^{-1} \times mg^{-1}$ protein.

The data of Table 1 show that FC does not significantly affect the initial rate of intravesicular acidification and the ATPase activity when the two activities are assayed at pH 6.6.

The activity of the PM H^+-ATPase presents a distinct pH optimum around pH 6.7 and sharply decreases with the increase of pH above the optimum value (14, Rasi-Caldogno et al. unpublished) so that at the cytoplasmic pH (7.3-7.6) its activity is far from being maximal.

The data in Table 1 show that, when the PM H^+-ATPase is assayed at pH 7.5, a stimulating effect of FC is evident both on intravesicular acidification and on phosphohydrolytic activity. No effect of FC on ATPase activity and on H^+-transport is evident in the presence of 100 μM vanadate which, in our experimental conditions, inhibits, the H^+-ATPase by 80%-90% (data not shown).

Fig. 1 shows that at pH 7.4 FC stimulates also Mg:ATP-dependent hyperpolaryzation of $\Delta\Psi$.

The effect of FC on the PM H^+-ATPase was investigated in a series of experiments run at pH 7.3-7.5 on various membrane preparations. Table 2 shows that the initial rate of Mg:ATP-dependent intravesicular acidification is strongly stimulated by FC in all cases; the extent of stimulation by FC varies from 50% to 130% depending on the membrane preparation utilized and/or on the slight variations of the pH of the medium.

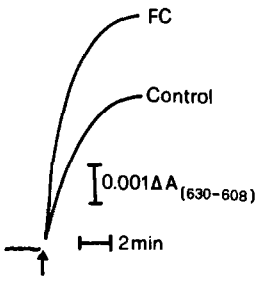

Figure 1. Effect of FC on Mg:ATP-dependent hyperpolaryzation of $\Delta\Psi$. Membrane vesicles were incubated as described in the Materials and Methods; ATP was added at the arrow.

A similar pattern was observed for FC-induced stimulation of the phosphohydrolytic activity. However the stimulating effect of FC on the phosphohydrolytic activity (from 20% to 45%) is always much lower than that on Mg:ATP-dependent intravesicular acidification.

In the pH range of these experiments (7.3-7.6) the activity of the PM H^+-ATPase is dramatically influenced by small changes in the pH of the incubation medium. FC does not affect the pH of the incubation medium (Table 2); this result supports the view that FC-induced stimulation of the PM H^+-ATPase reflects a direct activation of the enzyme by FC.

The lower effect of FC on the phosphohydrolytic activity than on H^+-transport driven by the PM H^+-ATPase might depend on various factors, such as: i) the presence in our microsomal fractions of other vanadate-sensitive ATP-hydrolyzing activities not affected by FC and not involved in H^+-transport; ii) the fact that FC might affect only those H^+-ATPase molecules which are properly inserted in the membrane; iii) the fact that FC might affect, beside the PM H^+-ATPase activity, the permeability of the PM to H^+. The last possibility seems unlikely in the light of the observation that FC does not affect the conductance to H^+ of the PM "in vivo" (15).

The results reported in this paper represent the first reliable demonstration that FC is able to stimulate the PM H^+-ATPase (ATP hydrolysis, ΔpH and $\Delta\Psi$ generation) in native membrane vesicles. FC-induced stimulation is evident at pH values (7.3-7.5) of physiological relevance and, at

Table 2. - FC-induced stimulation of Mg:ATP-dependent intravesicular acidification and of ATPase activity in various membrane preparations

membrane preparation	pH of the medium [a]		intravesicular acidification [b]		ATPase [c]	
	-	FC	-	FC	-	FC
1[d]	7.35	7.35	0.091	0.139	n.d.	n.d.
2	7.50	7.49	0.017	0.038	3.33	4.83
2[d]	7.41	7.41	0.027	0.049	n.d.	n.d.
3	7.45	7.46	0.041	0.062	3.07	3.63
4	7.45	7.45	0.017	0.031	2.77	3.48
5	7.52	7.53	0.032	0.060	3.74	4.80
5	7.35	7.35	0.089	0.132	n.d.	n.d.

a) - pH of the medium was measured sample by sample at the end of the intravesicular acidification measurements (3 to 5 minutes after ATP addition).

b) - $\Delta A_{(492-550)} \times min^{-1} \times mg^{-1}$ protein x ml.

c) - $\mu mol\ P_i \times h^{-1} \times mg^{-1}$ protein.

d) - Assays were run in the presence of 40 mM Hepes-Bistris instead of 40 mM Tris-Cl.

least when H^+-transport is measured, high enough to be compared with the "in vivo" effect of FC on H^+ extrusion.

In a recently appeared abstract, Cleland reported that FC lowers the apparent Km for MgATP of H^+ pumping into reconstituted pea root vesicles (16). Further work is necessary to elucidate the possible relationships between our results and those of Cleland.

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