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H^+-Pumping Driven by the Plasma Membrane ATPase in Membrane Vesicles from Radish: Stimulation by Fusicoccin[1]

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ABSTRACT

The effect of fusicoccin on Mg:ATP-dependent H^+-pumping in microsomal vesicles from 24-hour-old radish (*Raphanus sativus* L.) seedlings was investigated by measuring the initial rate of decrease in the absorbance of the ΔpH probe acridine orange. Fusicoccin stimulated Mg:ATP-dependent H^+-pumping when the pH of the assay medium was in the range 7.0 to 7.6 while no effect of fusicoccin was detected between pH 6.6 and pH 6.0. Both basal and fusicoccin-stimulated H^+-pumping were completely inhibited by vanadate and almost unaffected by nitrate. Fusicoccin did not change membrane permeability to protons and fusicoccin-induced stimulation of Mg:ATP-dependent H^+-pumping was not affected by changes in the buffer capacity of the incubation medium. Deacetylfusicoccin stimulated H^+-pumping as much as fusicoccin, while the physiologically inactive derivative 8-oxo-9-epideacetylfusicoccin did not. Stimulation of H^+-pumping was saturated by 100 nanomolar fusicoccin. These data indicate that fusicoccin activates the plasma membrane H^+-ATPase by acting at the membrane level independently of the involvement of other cell components. The percent stimulation by fusicoccin was the same at all ATP concentrations tested (0.5–5.0 millimolar), thus suggesting that with fusicoccin there is an increase in V_{max} of the plasma membrane H^+-ATPase rather than a decrease in its apparent K_m for Mg:ATP.

All of the physiological responses to FC[2] appear to be related to a stimulation of electrogenic H^+ extrusion mediated by the plasma membrane H^+-ATPase. Stimulation of the plasma membrane H^+-ATPase has been proposed as a primary step in the action of the toxin on the basis of evidence such as lack of a lag in the stimulating effect of FC on H^+ extrusion and on hyperpolarization of ΔΨ, insensitivity of FC effects to inhibitors of RNA and protein synthesis, identification of specific FC-binding proteins at the plasma membrane and some stimulation by FC of the ATPase activity measured in microsomal fractions (for a review, see Refs. 17 and 18).

In contrast to this view, it has been recently reported that FC induces in root hairs and in coleoptiles an early acidification of the cytoplasm, evident before any hyperpolarization of ΔΨ or stimulation of H^+ extrusion is detectable (5, 7, 15). Since it has been shown that electrogenic H^+ extrusion *in vivo* (6, 15, 28) as well as the plasma membrane H^+-ATPase *in vitro* (10, 14, 20, 29) are strongly stimulated by small decreases in pH in the range of cytoplasmic pH values, these data have been interpreted as indicating that FC-induced stimulation of electrogenic H^+ extrusion is a consequence of the acidification of the cytoplasm (5, 7, 15).

However, no significant effect of FC on cytoplasmic pH was detected by Guern *et al.* (13) and Roberts *et al.* (27), while a FC-induced alkalinization of the cytoplasm has been reported by Marrè *et al.* (19) and by Reid *et al.* (26). Moreover, Romani *et al.* (28) have shown that the effects of FC on H^+ extrusion, K^+ uptake, and hyperpolarization of ΔΨ are clearly synergistic with those of weak acids which decrease the cytoplasmic pH, thus strongly suggesting that the two agents influence the H^+-pump through different mechanisms. While the FC-induced change of the cytoplasmic pH remains open to investigation, it seems very unlikely that it represents the primary event leading to the stimulation of H^+ extrusion.

A more direct insight into the mechanism of action of FC can be provided by the study of its effects *in vitro*. This approach allows FC effects on membrane bound activities to be studied in the absence of other cell components. Recently, Rasi-Caldogno and Pugliarello (25), showed that FC strongly and reproducibly stimulated the H^+-pumping and, to a lesser extent, the hydrolytic activity of the plasma membrane H^+-ATPase in membrane vesicles isolated from radish seedlings. In this paper, we present a more detailed characterization of the stimulating effect of FC on the H^+-pumping activity of the plasma membrane H^+-ATPase of radish. The results clearly indicate that FC specifically activates the plasma membrane H^+-ATPase by acting at the membrane level.

MATERIALS AND METHODS

Preparation of Membrane Vesicles. Microsomal vesicles from 24-h-old radish seedlings (*Raphanus sativus* L. cv Tondo Rosso Quarantino, Ingegnoli, Milano, Italy) prepared as previously described (24) were suspended in 0.25 M sucrose, 0.2% BSA, 0.5 mM β-mercaptoethanol, 1 mM BisTris-Hepes (pH 7.0) at a concentration of 2 to 3 mg membrane protein ml^{-1} and stored at −70°C.

Pretreatment with FC. All the experiments were carried out on membrane vesicles pretreated with or without FC as follows. Aliquots of freshly-thawed membrane suspension, diluted 1 to 20 times with suspension medium, were incubated at 25°C for 15 min with or without FC (5 μM unless otherwise specified) in the presence of 4.5 mM $MgSO_4$. Then the membrane suspensions were maintained in an ice bath for up to 4 h without significant changes in Mg:ATP-dependent H^+-pumping activity. Pretreatment of the membranes in the conditions described above was necessary to obtain maximal stimulation by FC of the H^+-pumping activity.

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[2] Abbreviations: FC, fusicoccin; ΔΨ, membrane potential difference; BisTris, 2-bis(2-hydroxyethylamino-2-(hydroxymethyl)-1,3-propanediol; AO, acridine orange; BTP, bis-tris-propane (1,3-bis(tris-(hydroxymethyl)-methylamino)propane).

H^+-Pumping. Mg:ATP-dependent intravesicular acidification was monitored as the initial rate of decrease in AO absorbance ($\Delta A_{492-550}$) utilizing a dual wavelength Sigma ZWS II spectrophotometer. Membrane vesicles (about 0.07 mg membrane protein), pretreated with or without FC were incubated in 1.5 ml of a solution of 0.15 M KBr, 5 mM $MgSO_4$, 0.2 mM EGTA, 1.5 μM AO (recrystallized as previously described [24]), 40 mM BTP-Hepes, pH 7.55 (unless otherwise specified), and FC (at the same concentration as that during pretreatment) for 30 min at 33°C. In our experimental conditions, this treatment was necessary to equilibrate the intravesicular pH with the pH of the incubation medium (Fig. 3 and Table IV). The samples were then transferred to the spectrophotometer thermoregulated at 25°C. Following temperature equilibration (3–4 min), the reaction was started by the addition of ATP (3 mM unless otherwise specified). The pH values of the assay media were carefully adjusted to the desired pH values (±0.005) at 25°C with a Radiometer PHM 64 pH meter. Proton-pumping was quite constant in different membrane preparations, while FC-induced stimulation was somewhat more variable. Under standard assay conditions (pH 7.55) stimulation by saturating concentrations of FC ranged between 50 and 90%, depending on the membrane preparation utilized.[3] The reported results are the means of three or more experiments, each run with three replicates on at least two different membrane preparations. Standard deviations of the H^+-pumping measurements with the same membrane preparation did not exceed ±5 to 6%.

Kinetics of ΔpH Decay. Membrane vesicles (about 0.1 mg membrane protein) pretreated with or without 5 μM FC were diluted in 1.5 ml of a solution of 0.15 M KBr, 5 mM $MgSO_4$, 0.2 mM EGTA, 3 μM AO, 40 mM Mes-BTP pH 6.7 with (FC-pretreated samples) or without (controls) 5 μM FC. After equilibration at the specified temperatures, the medium was alkalinized to pH 7.5 by the addition of KOH and the time course of increase of AO absorbance was monitored for 15 to 30 min. The remaining ΔpH was then collapsed by addition of 10 mM $(NH_4)_2SO_4$. The decay of ΔpH was approximately exponential (see Fig. 3); half-times of ΔpH decay were computed by linear regression analysis as described by Lew and Spanswick (16).

Protein. Membrane protein was determined as previously described (11).

Chemicals. FC and deacetylfusicoccin, chromatographically pure, were kindly given by Dr. M. Radice, Montedison SpA, Milan, Italy; 8-oxo-9-epideacetylfusicoccin was a kind gift from Prof. A. Ballio, University of Rome. All other chemicals were analytical grade.

RESULTS

In microsomal vesicles from 24-h-old radish seedlings, Mg:ATP-dependent H^+-pumping, measured at pH 6.6, is completely inhibited by vanadate and resistant to nitrate, indicating that it is driven only by the H^+-ATPase of the plasma membrane (24). We previously reported that FC was able to stimulate Mg:ATP-dependent H^+-pumping in this membrane fraction; its effect was very marked when the assays were run at physiological pH values (7.4–7.6), while no stimulation could be observed at pH 6.6 (25).

Figure 1 shows the pH dependence of Mg:ATP-dependent H^+-pumping in microsomal vesicles from 24-h-old radish seedlings, treated with or without fusicoccin. Proton-pumping is strongly influenced by pH in the range from 6.0 to 7.6 and reaches a maximum around pH 6.6, thus confirming the strong pH dependence of the plasma membrane H^+-ATPase of plant cells (10, 14, 20, 29). No stimulation by FC is detectable at pH values 6.0 to 6.6, while a clear-cut stimulation is evident at pH values ranging from 7.0 to 7.6. FC-induced stimulation of H^+-pumping, computed on an absolute basis, decreases with the decrease of activity brought about by the increase of pH, while the percent effect of FC (inset to Fig. 1) increases with pH, reaching a value of about 80% at pH 7.6.

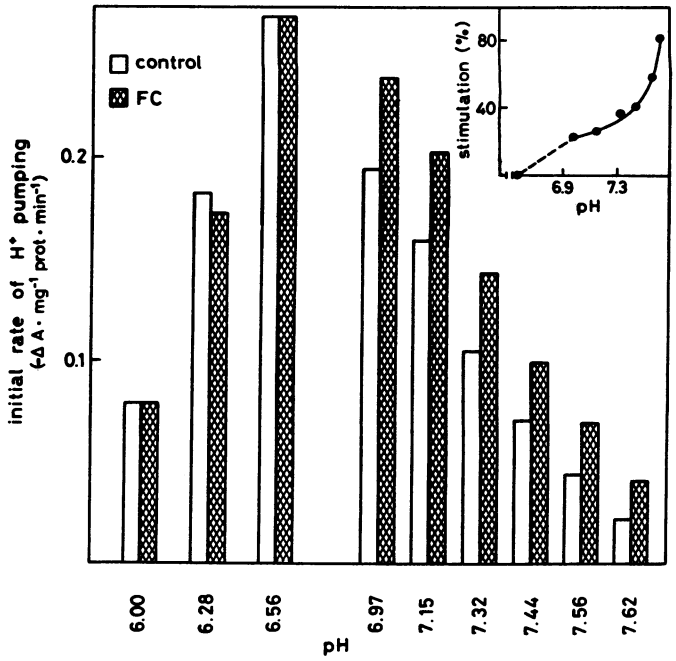

FIG. 1. Effect of the pH of the medium on Mg:ATP-dependent H^+-pumping in membrane vesicles treated with or without FC. Experimental conditions were as described in "Materials and Methods" except that the incubation medium was buffered at the specified pH values with 40 mM Mes-BTP (pH 6.00 to 6.97) or 40 mM BTP-Hepes (pH 6.97 to 7.62). pH values of the assay media were carefully adjusted to the specified values ± 0.005 at 25°C. Activity at pH 6.97 was the same with both the buffers used. Percent stimulation by FC is shown in the inset.

All the following experiments were carried out at pH 7.55, where FC stimulates Mg:ATP-dependent H^+-pumping by 50 to 90%, depending on the membrane preparation used. At higher pH values, H^+-pumping in the absence of FC was hardly measurable. It is worth noting that these results were obtained by pretreating the membrane suspension with FC in the presence of 4.5 mM $MgSO_4$, as described in "Materials and Methods." Lack of this pretreatment resulted in a less pronounced response to FC and increased the variability among different membrane preparations (data not shown). The reasons for this pretreatment requirement are at present being investigated in our laboratory.

When assayed at pH 7.55, the activity of the vanadate-sensitive H^+-ATPase of the plasma membrane is far from its maximal activity (about 20% of that measured at pH 6.6) while the activity of the nitrate-sensitive H^+-ATPase of the tonoplast is nearly maximal (see for example, Refs. 14, 29, 30). Therefore a very small contamination of the microsomal fraction by tonoplast H^+-ATPase, undetectable at pH 6.6 (24), could be important at higher pH values. To check this possibility, we measured the effects of vanadate and nitrate on Mg:ATP-dependent H^+-pumping at pH 7.55. Table I shows that H^+-pumping measured at pH 7.55 is completely inhibited by vanadate, ruling out any important contribution by the tonoplast H^+-ATPase to the H^+-pumping activity in this membrane fraction also at high pH values.

[3] After the completion of this work we found that the variability of the response to FC was greatly reduced by using freshly prepared extraction media to prepare the microsomal vesicles. Stimulation by FC of H^+-pumping in standard assay conditions on six membrane preparations prepared as described above was 112 ± 8%.

Table I. *Effects of Vanadate and of Nitrate on Mg:ATP-Dependent H⁺-Pumping in Membrane Vesicles Treated with or without FC*

Assays were carried out as described in "Materials and Methods."

Addition	Initial Rate of H⁺-Pumping		Effect of FC
	Control	FC	
	$-\Delta A\ mg^{-1}$ protein min^{-1}		
0.15 M KBr	0.040	0.065	+0.025
0.15 M KBr + 0.10 mM vanadate	0.001	0.001	0.000
0.10 M KBr + 0.05 M KNO_3	0.032	0.055	+0.023

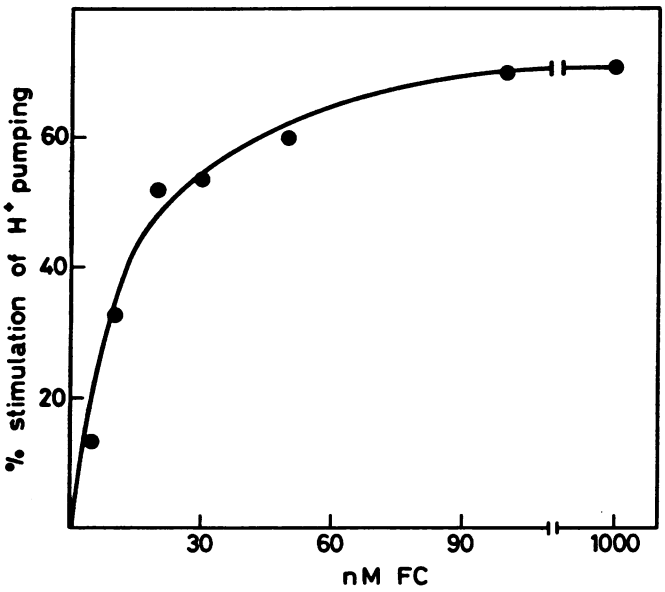

FIG. 2. Kinetics of FC-induced stimulation of Mg:ATP-dependent H⁺-pumping as a function of FC concentration. Membrane vesicles diluted 20 times with suspension medium were pretreated with FC at the concentrations specified. H⁺-pumping assays were carried out under standard conditions in the presence of the same concentration of FC as that applied during the pretreatment.

The finding that H⁺-pumping in the presence of nitrate is slightly lower than in its absence might reflect a backflow of H⁺ in symport with nitrate (29) (MI De Michelis, unpublished data). Table I shows also the effect of FC on H⁺-pumping: stimulation by FC is suppressed by vanadate and virtually unaffected by nitrate.

FC-induced stimulation of H⁺-pumping exhibits saturation kinetics (Fig. 2): stimulation is clearly detectable at a FC concentration as low as 5 nM and saturation is reached at 100 nM FC. These concentrations are somewhat lower than those able to induce maximal stimulation of electrogenic H⁺ extrusion in intact tissues but of the same order of magnitude as those able to saturate FC-binding proteins identified in isolated membrane fractions from other plant materials (1, 17, 21, 23).

The availability of derivatives of FC whose biological activities have been described (2, 3), allowed us to investigate whether the structural requirements for FC-induced stimulation of H⁺-pumping in isolated membrane vesicles resemble those required for the stimulation of *in vivo* H⁺ extrusion and for *in vitro* binding to the receptor. Table II shows the effect of FC and of two of these derivatives (deacetylfusicoccin and 8-oxo-9-epideacetylfusicoccin) on Mg:ATP-dependent H⁺-pumping. Deacetylfusicoccin, which stimulates H⁺ extrusion *in vivo* and competes with FC for its specific receptors (2, 3, 23), stimulates H⁺-pumping to

Table II. *Effects of FC and of Two of Its Derivatives on Mg:ATP-Dependent H⁺-Pumping*

Membrane vesicles were pretreated with the different substances as described in "Materials and Methods" and H⁺-pumping was assayed under standard conditions in the presence of the same substance applied during the pretreatment.

Addition	Initial Rate of H⁺-Pumping	Stimulation over Control
	$-\Delta A\ mg^{-1}$ protein min^{-1}	
Control	0.046	
1 μM FC	0.075	+0.029
1 μM Deacetylfusicoccin	0.073	+0.027
1 μM 8-Oxo-9-epideacetylfusicoccin	0.050	+0.004
20 μM Indole-3-acetic acid	0.047	+0.001

Table III. *Lack of Influence of Buffer Capacity of the Medium on FC-Induced Stimulation of Mg:ATP-Dependent H⁺-Pumping*

Assays were carried out under standard conditions, but the medium was buffered with 8 or 80 mM BTP-Hepes (pH 7.55).

Buffer Concentration	Initial Rate of H⁺-Pumping		Effect of FC
	Control	FC	
mM	$-\Delta A\ mg^{-1}$ protein min^{-1}		
8	0.043	0.075	+0.032
80	0.038	0.065	+0.027

the same extent as FC, while 8-oxo-9-epideacetylfusicoccin, an inactive derivative (2), is virtually ineffective. The natural hormone indole-3-acetic acid has no effect on H⁺-pumping activity in our experimental system.

The findings that the FC effect is saturated at very low FC concentrations and that deacetylfusicoccin is as effective as FC, rule out the hypothesis suggested by Hager and Moser (15) that the effect of FC is mediated by an acidification of the environment of the plasma membrane H⁺-ATPase due to the cleavage of the acetyl groups of FC by esterase activities.

Fusicoccin-induced stimulation of H⁺-pumping might reflect an acidification of the environment of the plasma membrane H⁺-ATPase somehow else induced by FC. If this were the case, FC-induced stimulation of H⁺-pumping should be strongly influenced by changes in the buffering capacity of the assay medium. Table III shows that the effect of FC is virtually unaffected by raising the concentration of the buffer from 8 to 80 mM.

The stimulating effect of FC on H⁺-pumping might reflect, rather than an activation of the enzymic machinery, a change in the permeability of the membrane to H⁺ induced by the toxin. We checked this possibility by investigating the effect of FC on the time course of the decay of an imposed pH gradient. Figure 3 shows that the ΔpH generated by a sudden alkalinization of the incubation medium decays in a roughly exponential fashion both at 25°C and at 33°C. The half-times of ΔpH decay, calculated by linear regression analysis (16), are about 26 min at 25°C and 10 min at 33°C (Table IV). No effect of FC is evident on the half-time of the ΔpH decay at both temperatures, thus indicating, in agreement with the results of *in vivo* experiments (12, 22), that FC does not affect the permeability of the membrane to H⁺. These results together with our previous finding that FC stimulates the hydrolytic activity of the plasma membrane H⁺-ATPase (25) indicate that FC-induced stimulation of Mg:ATP-dependent H⁺-pumping depends on a true activation of the plasma membrane H⁺-ATPase.

Cleland has recently reported (8) that FC lowers the K_m for

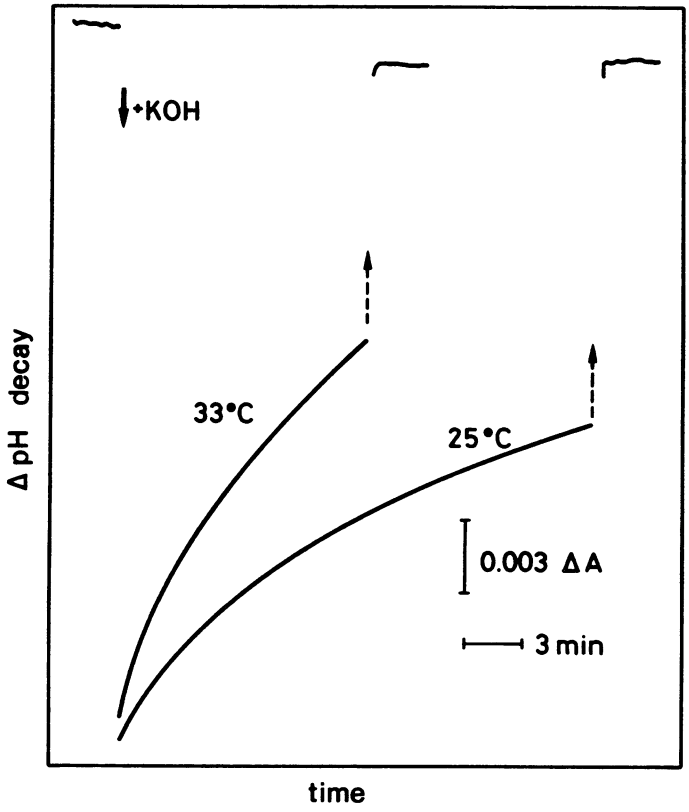

FIG. 3. Time course of decay of an imposed pH gradient. Membrane vesicles (0.07 mg membrane protein ml^{-1}) were equilibrated in 0.15 M KBr, 0.2 mM EGTA, 5 mM $MgSO_4$, 3 μM AO, 40 mM Mes-BTP (pH 6.7) at the specified temperatures until a steady baseline of AO absorbance was reached. The medium was then alkalinized to pH 7.5 by addition of KOH (first arrow) and the time course of AO release was monitored. $(NH_4)_2SO_4$ (10 mM) was added at the dashed arrows to dissipate the remaining ΔpH.

Table IV. *Lack of Effect of FC on the Permeability of Membrane to H^+*

Membrane vesicles pretreated with or without FC were incubated as described in the legend to Figure 3. Half-times of ΔpH decay were computed by linear regression analysis as described by Lew and Spanswick (16): regression coefficients were 0.98 or higher.

Temperature	Half-Time of ΔpH Decay	
	Control	FC
°C	*min*	
25	26.1 ± 1.1	27.1 ± 1.3
33	10.0 ± 0.4	9.7 ± 0.7

Mg:ATP of H^+-pumping driven by the plasma membrane H^+-ATPase in reconstituted pea root vesicles. We measured the effect of FC on H^+-pumping at various ATP concentrations; Figure 4 shows that Mg:ATP-dependent H^+-pumping increases with ATP concentration up to 2.5 mM ATP, then seems to reach saturation both in the presence and in the absence of FC. Although a kinetic analysis of these data is not possible because of some interference between acridine orange and ATP (F Rasi-Caldogno, MI De Michelis, MC Pugliarello, unpublished data), the finding that the percent stimulation of H^+-pumping by FC is roughly the same at all the ATP concentrations tested shows that the stimulation of H^+-pumping by FC in our experimental system does not depend on a decrease of K_m for ATP.

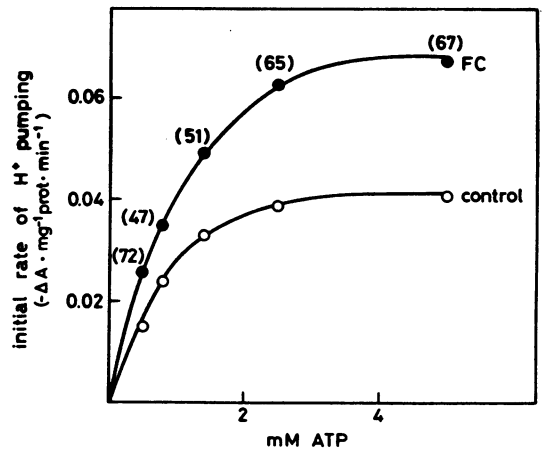

FIG. 4. Effect of FC on Mg:ATP-dependent H^+-pumping in the presence of various concentrations of ATP. Assays were carried out under standard conditions but $MgSO_4$ concentration was raised to 10 mM and ATP concentration was varied between 0.5 and 5.0 mM. Numbers in brackets represent percent stimulation by FC.

DISCUSSION

Understanding the mechanism by which FC activates electrogenic H^+ extrusion in plant cells is very important for elucidating the sequence of metabolic effects induced by FC and by natural hormones, as well as the role of the plasma membrane H^+-ATPase in the modulation of cell metabolism. In the last 10 years many attempts have been made in this and other laboratories to demonstrate a direct effect of FC on the plasma membrane H^+-ATPase in membrane fractions, but the results obtained were either not very reproducible or contradictory (*e.g.* 4, 9, 18). The success of this investigation in demonstrating that FC stimulates the H^+-pumping activity as well as the hydrolytic activity (25) of the plasma membrane H^+-ATPase in microsomal vesicles from radish seedlings depends on the fact that our experiments were carried out at pH 7.5 rather than in the pH range (6.4–6.8) for maximal activity of the plasma membrane H^+-ATPase (10, 14, 20, 29). In the latter pH range no stimulation by FC is detectable in our system (Fig. 1). It is worth noting that FC-induced stimulation of H^+-pumping driven by the plasma membrane H^+-ATPase is evident in the cytoplasmic pH range of higher plant cells (pH 7.2–7.6) (26, 27).

The findings that FC also stimulates the hydrolytic activity of the plasma membrane H^+-ATPase (25) and does not affect the permeability of the membrane to H^+ (Table IV) (12, 22) indicate that FC-induced stimulation of Mg:ATP-dependent H^+-pumping reflects a true activation of the plasma membrane H^+-ATPase. This activation is not the result of a FC-induced decrease of pH in the environment of the strongly pH-sensitive enzyme since FC does not induce any measurable acidification of the incubation medium (25) and the response to FC is unaffected by changes in the buffer capacity of the incubation medium (Table III). Diversely from what reported by Cleland (8), stimulation by FC of H^+-pumping driven by the plasma membrane H^+-ATPase reflects an increase of V_{max} rather than a decrease of the apparent K_m for Mg:ATP (Fig. 4). The effect of FC on Mg:ATP-dependent H^+-pumping displays a dependence on its concentration (Fig. 2) similar to that of the binding of the toxin to membrane bound specific receptors (1, 21, 23). Moreover the structural requirements for FC-induced activation of H^+-pumping (Table II) are similar to those for binding to the receptor as well as for *in vivo* physiological responses (2, 3, 23).

The data reported in this paper support the view that the FC-induced activation of the plasma membrane H^+-ATPase at the membrane level is the primary event leading to the metabolic

and physiological effects induced by FC *in vivo*.

The relationships between binding to the receptor and activation of the plasma membrane H^+-ATPase must be further investigated in order to ascertain the mechanism through which the binding of FC to the receptor determines an activation of the plasma membrane H^+-ATPase and whether the receptor and the ATPase are the same or different proteins.

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