Evidence for Dual Sites of Fusicoccin-Mediated Plasmalemma Proton Efflux[1]

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ABSTRACT

The effects of fusicoccin (FC), diethylstilbestrol (DES), *N, N'*-dicyclohexyl carbodiimide (DCCD), oligomycin, and *p*-trifluoroethyoxy carbonyl cyanide phenylhydrazone (FCCP) were studied on K^+ and H^+ fluxes, plasmalemma ATPase activity, respiration, and ATP concentration in fresh and washed corn root segments. DES strongly inhibited ion fluxes and ATPase activity. At high concentrations, DES showed strong uncoupling effects. DES caused a transient increase in cellular ATP content which was followed by a marked reduction. At nonuncoupling concentrations, DES as well as DCCD and oligomycin only slightly inhibited the FC-stimulated H^+ efflux while completely inhibiting the basal H^+ efflux. Plasmalemma ATPase activity isolated from washed, but not from fresh tissue, was stimulated 20% by FC and was sensitive to DES. These data suggest two plasmalemma-H^+ extrusion systems, one is ATPase mediated and the other, which is the primary site of FC stimulation, is not ATPase mediated.

INTRODUCTION

Fucicoccin (FC), a diterpene glucoside phytotoxin, is known to catalyze an active, electrogenic H^+/K^+ exchange in many plant tissues (Cleland, 1976; Marrè, 1977[a]; Marrè, 1977[b]; Marrè, 1979; Pitman, 1975). The rapid kinetics of stimulation of H^+ extrusion and cation (mainly K^+) uptake, and hyperpolarization of the transmembrane potential difference (PD) by FC are consistent with FC acting at the plasmalemma (Cleland *et al.*, 1977; Marrè, 1977[a]). Although the mechanism by which FC stimulates ion transport and PD is not known, it has been proposed that FC activates a plasmalemma ATPase which mediates an electrogenic H^+/K^+ antiporter (Cleland, 1976; Marrè, 1977[a]; Marrè, 1977[b]; Marrè, 1979; Pitman, 1975). Evidence supporting this model is the binding of radiolabeled FC to a plasmalemma-enriched membrane fraction (Dohrmann *et al.*, 1977; Stout and Cleland, 1979), and the stimulation of *in vitro* plasmalemma ATPase activity. However, the consistency of the stimulation of *in vitro* ATPase activity by FC is unsatisfactory and as indicated by Marrè (1979) these lines of evidence are far from conclusive.

Another possibility is that FC does not act solely by stimulating the plasmalemma ATPase

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2. Abbreviations: DCCD: *N, N'*-dicyclohexylcarbodiimide;
DES: diethylstilbestrol;
FC: fusicoccin;
FCCP: (*p*-trifluoromethoxy) carbonylcyanide phenyldrazone;
PCMBS: *P*-chloromercuribenzenesulfonic acid;
PD: electrical potential difference between vacuole and external solution.

"proton" pump, but instead activates a non-ATPase mediated H^+/K^+ exchange. In this report, we show that DCCD, DES, and oligomycin, all inhibitors of ATPase activity, strongly inhibited the basal but not the FC-stimulated proton efflux in corn root tissue. These data indicate that FC does not act solely by stimulating the plasmalemma ATPase-mediated "proton-pump" but instead activates a separate non-APase mediated proton-potassium exchange.

MATERIALS AND METHODS

Methods for growing corn seedlings (*Zea Mays* L. B73X Missouri 17) and cutting and washing root segments for 4 h were described previously (Lin, 1979). Measurements of K^+ and H^+ fluxes were essentially as that described by Lin (1979) but without any pretreatment with compounds prior to the assays. About 0.15 g of root segments was used for ATP extraction (Lin and Hanson, 1974) and ATP concentration was then measured immediately with a Du Pont Luminescence Biometer. Respiration was determined polarographically at 30°C using twenty 1-cm root segments (about 0.15 g) (Lin and Hanson, 1976).

FC was a generous gift from Professor E. Marrè, Milan, Italy. DCCD, DES, and oligomycin were purchased from Sigma Chemical Company. All other chemicals were ACS reagent grade.

RESULTS AND DISCUSSION

DES and DCCD, both inhibitors of plasmalemma ATPase activity (Balke and Hodgs 1979; Bowman *et al.*, 1978) and oligomycin, an inhibitor of mitochondrial ATPase (Bowman *et al.*, 1978) and probably of plasmalemma ATPase (Beffagna *et al.*, 1977; Malone *et al.*, 1977) were used to investigate the nature of the FC stimulation of net proton efflux. It is widely assumed that higher plant cells display an active proton extrusion driven by a vectorial plasmalemma ATPase (Hodge, 1976). This ATPase-mediated proton efflux into a potassium-containing medium was used to study the FC-stimulated proton efflux in relation to membrane ATPase activity.

Figure 1 shows that low concentrations of DES (<25-30 μM) markedly inhibited net basal proton efflux without affecting the FC-stimulated proton efflux in both fresh and washed

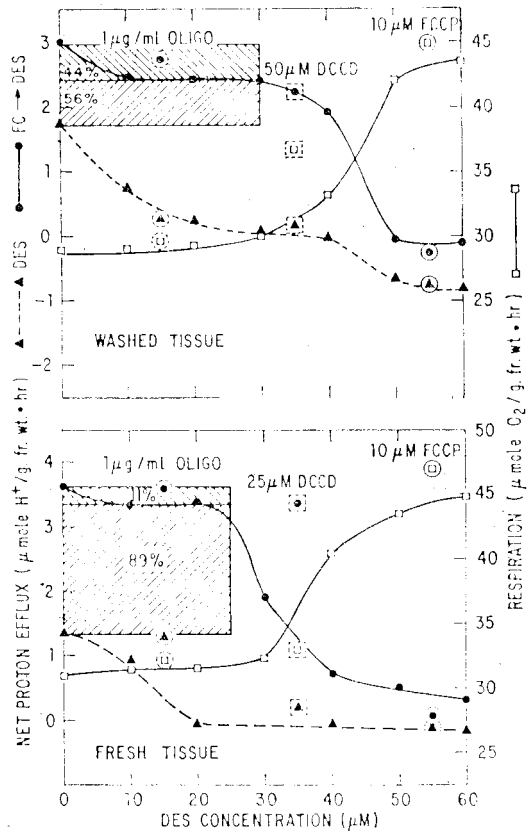

Fig. 1. Effects of DES, DCCD, oligomycin(OLIGO), and FCCP on corn root respiration (■), basal (▲) and FC-stimulated (•) net H^+ efflux. FC concentration was 20 μM. In fresh tissue (lower panel) 11% of FC-stimulated net H^+ efflux was DES sensitive, and 89% was DES insensitive. In washed tissue (upper panel) it was 44% and 56%, respectively. Points outlined with closed circles, dashed square and dashed circles represent the effect of FCCP, DCCD and oligomycin, respectively, in the absence of DES.

corn root segments. At higher concentrations (>30 μM) DES inhibited the FC-stimulated rate but this inhibition can be attributed to an uncoupling effect since these DES concentrations increased tissue respiration and inhibited basal proton efflux to the same extent as 10 μM FCCP (symbols outlined with closed circles). Similarly, low concentrations of DCCD (symbols in dashed square) and olgomycin (symbols in dashed circles), in the absence of DES, also strongly inhibited the net basal but not the the FC-stimulated proton efflux.

In washed tissue, in the absence of DES, 20 μM FC stimulated the net basal proton efflux by approximately 75% (from 1.7 to 3 μmol H^+/g fresh wt). That the FC-stimulated H^+ efflux was only partially (46%) inhibited by DES at a nonuncoupling concentration which completely inhibited basal H^+ efflux suggests that 56% of the FC-stimulated rate was non-ATPase mediated. In fresh tissue, FC stimulated the basal rate by about 190% and only 11% of this FC-stimulated rate was sensitive to DES when used at nonuncoupling concentrations. That the uncoupler, FCCP, completely inhibited the DES, DCCD, or oligomycin insensitive FC-stimulated rate indicates that this net proton efflux is metabolic.

Interestingly, in fresh tissue the net H^+ efflux is insensitive to oligomycin (Figure 1) while in washed tissue oligomycin almost completely inhibited the basal H^+ efflux and partially inhibited the FC-stimulated efflux suggesting that washing restores a portion of efflux component which was lost due to the injury by root cutting (Gronewald and Hanson, 1979).

At nonuncoupling concentrations, DES strongly inhibited the basal K^+ uptake in both fresh and washed tissue by 70% (Table I), whereas FC increased K^+ uptake by 100 and 40% in fresh and washed tissue, respectively. When DES was added after the tissue was added after the tissue was treated with FC for for 15 min, a substantial amount of K^+ was still accumulated into the tissue. When the tissue was first treated with DES no further accumulation of K^+ occurred, even when FC was added. A similar result was observed for net H^+ efflux. The inability of FC to overcome the DES inhibition of K^+ and H^+ transport probably is due to an inhibition of cellular energy metabolism (Balke and Hodge, 1977; Balke and Hodge, 1979). This view is supported by the effects of DES on root ATP concentration. Both DES and FC caused a transient increase in ATP concentration followed by a decrease in both fresh and washed tissue (data not shown). Transient increases of ATP by DES may result from an initial inhibition of plasmalemma ATPase activity and reduced the

Table I. Effect of FC and DES on potassium (^{86}Rb) uptake in Corn Root Tissue

	Fresh Tissue (nmol/g fwt•min)	(%)	Washed Tissue (nmol/g fwt•min)	(%)
control	7.2	100	43.7	100
FC (20 μM)	16.2	225	61.3	140
DES (20 μM)	2.2	31	13.1	30
FC→DES*	9.0	125	38.3[a]	88
			29.0[b]	44

All uptake period was 15 min with the exception of "FC→DES" group as indicated.
* FC was added 15 min prior to DES.
a first 15 min after the addition of DES.
b second 15 min after the addition of DES.

ATP consumption rate. The subsequent decrease of ATP concentration most probably reflects the DES inhibition of cellular energy metabolism (Balke and Hodge, 1977; Balke and Hodge, 1979). A similar inhibition of ATP concentration has been reported in oat roots (Balke and Hodge, 1979). If FC is stimulating plasmalemma ATPase activity, ATP consumption rate should be increased and the ATP concentration inside the tissue would consequently be lower. The transient increase of ATP concentration in FC treated tissue suggests that ATPase activity was inhibited initially. However, the results presented here and elsewhere (Beffagna *et al.*, 1977; Cleland, 1976; Gronewald, 1979; Marrè, 1979) argue against the possibility that FC is inhibiting ATPase activity. Nevertheless, transient increase followed by a decrease of ATP concentration in tissue by the treatment of DES and FC further supports the hypothesis that ATPase is not the major FC acting site. Recently a plasmalemma redox electron transport chain (Novak and Ivankina, 1978) has been suggested to be involved in the active H^+ pump. Studies on the effect of FC on this H^+ pump or the binding of FC to components of this redox chain would provide further insights into the mode of action of FC.

As stated in the Introduction, the stimulation of *in vitro* ATPase by FC is quite inconsistent and thus not easily interpreted. Table II indicates that the FC stimulation effect is dependent on the physiological state of the tissue. No significant effect of FC was found on plasmalemma ATPase activity isolated from freshly harvested tissue whereas plasmalemma ATPase activity from washed tissue (4 hr) was routinely increased by 20%. DES strongly inhibited ATPase activity irregardless of washing or the presence of FC. These results are inconsistent with the FC effect on K^+ and H^+ fluxes. FC stimulated net H^+ efflux (8 and Figure 1) and K^+ uptake (Gronewald *et al.*, 1979) fresh tissue to a greater extent than that of washed tissue. Unless H^+ and K^+ fluxes are not linked to plasmalemma ATPase activity, the site of action of FC on the plasmalemma does not seem to be at ATPase site. In this regard the separation of a FC binding site distinct from ATPase plasmalemma preparations has been reported recently (Marrè, 1979).

Both fusicoccin and auxin promote growth in a variety of plant cells. Although there are striking similarities between these two growth-promoting agents, such as the simultaneous activation of H^+ efflux, K^+ uptake, and PD hyperpolarization (Marrè, 1979), it is not known whether FC and auxin act at the same H^+/K^+ exchange system. Further study of the effects of FC and auxin on ion transport may resolve this question and, indeed, may provide insight into the broader areas of hormonal control of ion transport and its role in cell growth.

Table II. Effect of FC and DES on Plasmalemma ATPase Activity Isolated from Corn Root Tissue

	Fresh Tissue (μmol Pi/mg protein hr)	(%)	Washed Tissue (μmol Pi/mg protein hr)	(%)
Control	30.27	100	33.33	100
FC (20 μM)	27.07	90	40.00	120
DES (50 μM)	17.63	58	22.17	67

Reaction mix consisted of 3 mM ATP, 1.5 mM $MgSO_4$, 33 mM tris-MES (pH 6.0), and 50 mM KCl, and 35 to 40 μg protein. Reaction time was terminated at 15 min with cold 5% TCA.
Mg^+-ATPase activity was 21.33 and 23.21 μmol Pi/mg•protein•hr for fresh and washed tissue, respectively.

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Fusicoccin 導致細胞膜質子流出作用具有雙作用位置的證明

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摘 要

FC, DES, DCCD, Oligomycin 和 FCCP 等用來研究對新鮮的處理過的玉米根的 H^+ 和 K^+ 的 Plasmalemma-ATPase 流動、的活動力、呼吸作用、以及 ATP 的濃度等四種生理作用的影響。

DES 高度抑制離子 (K^+ 及 H^+) 的流動及 ATPase 的活動力，在高濃度下，DES 顯示出高度的 Uncoupling 作用。DES 引起細胞內 ATP 含量暫時性的增加隨顯著降低。在 NON-uncoupling 濃度下，DES, DCCD 和 Oligomycin 等只輕微抑制 FC-Stimulated H^+ 的流出（細胞外）而完全抑制 Basal H^+ 的流出。

FC 可以增加 20% Plasmalemma ATPase 的活動力（處理過的玉米根分離出來），但這種增加可以完全被 DES 抵消掉。

以上的結果指出有兩種 Plasmalemma-H^+ 流出系統，一種是介由 ATPase，另一種是 FC 主要的 Stimulated site，並不介由 ATPase。