Plant Physiol. (1980) 66, 1095-1098
0032-0889/80/66/1095/04/$00.50/0

Relationship between ATP Level and Activity of Fusicoccin-stimulated H^+/K^+-Exchange System in Plant Tissues

Received for publication August 20, 1979 and in revised form July 7, 1980

FRANCA RASI-CALDOGNO, RAFFAELLA CERANA, AND MARIA C. PUGLIARELLO
Centro di Studio del Consiglio Nazionale delle Ricerche per la Biologia Cellulare e Molecolare delle Piante, Istituto di Scienze Botaniche, Università di Milano, Italy

ABSTRACT

The treatment with fusicoccin causes a slight but significant decrease (about 15%) in the ATP level in pea-internode and maize-coleoptile segments. This decrease is detectable within 15 minutes and is accompanied by a parallel increase in O_2 uptake. Sodium azide inhibits O_2 uptake and completely blocks the stimulation of O_2 uptake by fusicoccin in both pea and coleoptile segments. Benzohydroxamic acid does not affect either basal or fusicoccin-induced O_2 uptake in maize-coleoptile sections. The drop of ATP level induced by various treatments (sodium arsenate, 2-deoxyglucose, limiting O_2, and anaerobiosis) is accompanied by a parallel inhibition of K^+ uptake in maize coleoptiles treated with or without fusicoccin. These results are consistent with the hypothesis that ATP is the energy source for the fusicoccin-activated H^+/K^+-exchange system.

The electrogenic proton extrusion stimulated by FC[1] is an energy-dependent process which is inhibited by respiratory and metabolic poisons (12, 16). Recent data indicate that, in *Neurospora* (18, 19) and in bacteria (6), ATP is the energy source for electrogenic transport systems presumably analogous to the one activated by FC in higher plants. Substantial evidence, although indirect, suggests that an ATPase in higher plants is also involved in H^+ extrusion and K^+ uptake (15). (*a*) There is a good correlation between K^+ uptake *in vivo* and K^+-stimulated ATPase activity *in vitro* in various plant materials (7). K^+ uptake and ATPase activity show similar kinetics as a function of K^+ concentration (7). (*b*) Inhibitors of ATPase activity *in vitro* inhibit also the H^+/K^+-exchange system *in vivo* (1, 15). (*c*) FC stimulates *in vivo* the H^+/K^+-exchange system (13) and activates *in vitro* a plasmalemma ATPase (2). (*d*) The binding of FC to the plasmalemma and the ATPase activity are equally inhibited, on a per cent basis, by two different inhibitors (diethylstilbestrol and *Cercospora beticola* toxin) (20).

If ATP provides the energy for H^+ and K^+ transport and FC stimulates an H^+-pumping ATPase, one would expect the stimulation of H^+ extrusion by FC to be paralleled by an increased rate of ATP utilization and, thus, by some drop of ATP/ADP + AMP ratio or, in general terms, of the energy charge. The drop of the energy charge would be expected to bring about an increase of Cyt oxidase-mediated O_2 uptake. Moreover, a correlation should exist between the changes of ATP level obtained with various treatments and the rate of proton extrusion and of K^+ uptake.

In the work reported here, the following have been studied: (*a*) the effect of FC on ATP level and on O_2 and K^+ uptake in pea internodes and maize coleoptiles; (*b*) the correlation between the drop in intracellular ATP level and the inhibition of the H^+/K^+-exchange system activity determined by various treatments in maize coleoptiles, treated or not with FC.

[1] Abbreviation: FC, fusicoccin.

MATERIALS AND METHODS

Maize (*Zea mays* L. cv. Dekalb XL 640) seeds and peak (*Pisum sativum* cv. Alaska) seedlings were grown for 4 and 6 days, respectively, at 26 C on poplar sawdust in the dark.

Maize-coleoptile segments, 3 mm long, were cut from the region between 5 and 15 mm from the tip; the segments were longitudinally divided in half. Pea-stem segments, 2 mm long, were cut in the subapical region of the distal internode. The segments were washed for 2 h in 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$ (the solution was changed after 30 min) and then transferred to the various media as described in the individual experiments; 0.5 mM $CaCl_2$ and 0.25 mM $MgCl_2$ were present in every treatment. The experiments were run in the dark in a thermoregulated water-bath with shaking (70 shakes/min) at 28 C in the case of maize coleoptiles and at 25 C in the case of pea-stem segments.

An atmosphere of 3% O_2 or anaerobiosis was obtained by continuous bubbling in the incubation medium of 3% O_2-97% N_2 or of O_2-free N_2.

Three different methods of ATP extraction from the tissue were tested: incubation in boiling triethanolamine HCl/NaOH buffer (pH 7.6) for 1 min (5), treatment with liquid N_2 and 5% trichloroacetic acid containing 0.05% 8-hydroxyquinoline, and treatment with 0.8 N $HClO_4$ at 0 C. The tissue was always homogenized in a mortar in the presence of sea sand. The highest values of ATP levels were obtained by extraction with 0.8 N $HClO_4$ at 0 C, therefore, this extraction was performed in all the experiments. The 3% O_2 atmosphere and anaerobiosis were maintained during fixation of the tissue with $HClO_4$. The homogenate was centrifuged for 5 min and the pellet was washed with 0.8 N $HClO_4$. The two supernatants were combined, neutralized with KOH in the presence of 120 mM triethanolamine HCl/NaOH buffer (pH 7.6), and centrifuged again. The enzymic assay of ATP was performed on the supernatant according to Lamprecht and Trautshold (10).

K^+ uptake was assayed by using rubidium-86 as a tracer. Incubation was performed in 1 mM KCl labeled with 0.5 μCi $^{86}RbCl$; at the end of the incubation, the tissue was briefly rinsed with 10 ml cold unlabeled solution and then washed at 0 C for 10 min in 5 ml ice-cold unlabeled solution; finally, the sections were rapidly rinsed with ice-cold H_2O. Distilled H_2O (4 ml) and 10 ml Instagel (Packard) were added to the tissue and the radioactivity of the samples was measured in a Packard Tri-Carb scintillation counter.

O_2 uptake was measured according to the usual techniques either in a Warburg microrespirometer or with a Clark O_2 electrode.

Pea-stem mitochondria were extracted at 0 C by homogenizing the tissue with 3 volumes of the following medium: 20 mM Hepes

(pH 7.6), 5 mM EDTA, 0.4 M sucrose, 0.1% BSA. The homogenate was filtered through four layers of cheesecloth and then centrifuged at 13,000*g* for 5 min. The pellet was resuspended in a glass Potter homogenizer with 30 ml of the extraction medium and then centrifuged at 2,500*g* for 3 min. The supernatant was centrifuged at 13,000*g* for 5 min and the pellet was resuspended in 0.4 M sucrose. The incubation medium during measurements of O_2 uptake by isolated mitochondria was 20 mM Hepes (pH 7.5), 0.4 M sucrose, 5 mM Na_2HPO_4, 5 mM $MgCl_2$, 10 mM succinate or malate.

Substrates and enzymes for enzymic assays were obtained from Boehringer.

RESULTS

Rapid Effects of FC on O_2 Uptake, ATP Level and K^+ Uptake. Figure 1a shows the time course for FC-induced stimulation of O_2 uptake in maize coleoptiles. The stimulating effect of FC is already detectable within 10 min and is maximum after 25 min. Similarly, the promoting effect of FC on K^+ uptake is very rapid, being detectable within 5 min of treatment with FC (Fig. 1b). Similar time courses are reported for FC-induced H^+ extrusion and hyperpolarization of transmembrane potential in maize roots and oat coleoptiles (3, 14). The stimulation of Q_{O_2} by FC is not peculiar to maize coleoptiles. In fact, Table I shows that FC also induces a strong increase of O_2 uptake in pea-internode segments, as already reported (12). Basal and FC-induced O_2 uptake is unaffected by benzyhydroxamic acid and strongly inhibited by NaN_3. Inhibition of NaN_3 on basal and FC-induced Q_{O_2} is very rapid (Fig. 2a), suggesting that, in both cases Q_{O_2} depends on the activity of the oxidative pathway involving Cyt oxidase. The data of Figure 2b show that FC does not influence Q_{O_2} of isolated

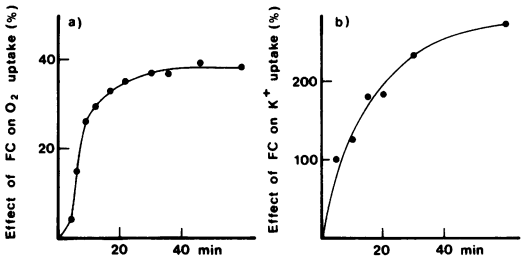

FIG. 1. Time courses for FC-stimulated O_2 and K^+ uptake. a, O_2 uptake was measured with a Clark O_2 electrode. Maize-coleoptile sections (50 mg/ml) were incubated in 1 mM KCl ± 20 μM FC for the indicated times. Data are expressed as per cent of FC stimulation over the control. b, maize-coleoptile sections (50 mg/ml) were incubated in 1 mM KCl + 0.5 μCi $^{86}RbCl$ ± 20 μM FC for the indicated times. Data are expressed as per cent of FC stimulation over the control.

Table I. *Effect of FC on Oxygen Uptake in Pea-stem and Maize-coleoptile Sections*

Q_{O_2} of pea-stem sections was measured with a Clark O_2 electrode; the sections (80 mg) were incubated in 2 ml 1 mM KCl ± 20 μM FC. Q_{O_2} of maize-coleoptile sections was measured with a Warburg microrespirometer; the sections (250 mg) were incubated in 2.5 ml 1 mM KCl ± 20 μM FC ± 0.5 mM NaN_3 ± 5 mM benzhydroxamic acid (BHAM).

Additions	O_2 Uptake			
	Pea-stem Sections	Maize-coleoptile Sections		
			NaN_3	BHAM
	μl g⁻¹ fresh wt h⁻¹			
None (control)	430	413	159	420
FC	614	566	154	572

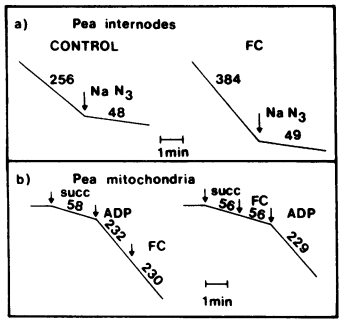

FIG. 2. a, Kinetics of NaN_3 inhibition of O_2 uptake in pea-stem sections. The O_2 uptake was measured with a Clark O_2 electrode. Pea-stem sections (80 mg) were incubated in 2 ml 1 mM KCl ± 20 μM FC; at the arrow, NaN_3 was added at the final concentration of 0.5 mM. The figures on the slopes = nmol O_2 g^{-1} fresh weight min^{-1}. b, Lack of effect of FC on O_2 uptake by isolated pea mitochondria. Q_{O_2} of mitochondrial suspension (0.2 ml) was measured in 2 ml of the incubation medium with a Clark O_2 electrode. Succinate (succ), ADP, and FC were used at the final concentrations of 10 mM, 1 mM and 20 μM, respectively. The figures on the slopes = nmol O_2 mg^{-1} protein min^{-1}.

Table II. *Effect of FC on ATP Level*

The segments were incubated in the presence of 1 mM KCl ± 20 μM FC for 15 or 60 min. The tissue to volume ratio was 100 mg/ml in the case of peak stem sections and 50 mg/ml in the case of maize coleoptiles. The figures are the means of six replicates for pea stem and of 18 replicates for maize coleoptiles ± SE.

Additions	Pea-stem Sections, 60 min	Maize-coleoptile Sections	
		15 min	60 min
	nmol g^{-1} fresh wt		
None (control)	79 ± 1.8	146 ± 2.3	140 ± 1.6
FC	67 ± 2.5	123 ± 1.4	119 ± 2.1

mitochondria in state 3 as well as in stage 4; the same behavior is observed when succinate is replaced by malate as respiratory substrate (data not shown). Thus, FC-induced stimulation of Q_{O_2} does not seem to depend on a direct effect of the drug on either the oxidative or the phosphorylative activities of the mitochondria.

Table II shows the ATP levels of pea internodes and maize coleoptiles incubated with or without FC. FC induces a small, but statistically highly significant, decrease of the ATP level (about 15%) in both materials. In maize coleoptiles, a significant decrease of ATP levels in the FC-treated samples is detectable after 15 min incubation with FC. Some effect of FC on ATP level is detectable after 5 and 10 min but, because of the scattering of the data, the difference is not statistically significant.

The stimulating effect of FC on Q_{O_2} seems conveniently explained by the fact that FC decreases ATP level (and, presumably, the energy charge) by promoting some energy-dependent, ATP-utilizing process. The earliness of the stimulation of Q_{O_2}, H^+ extrusion, and K^+ uptake suggests that FC accelerates the utilization of ATP by an energy-dependent H^+/K^+-exchange system.

ATP Levels and K^+ Uptake. Previous data, obtained in pea-stem and maize-coleoptile segments, indicate that various treatments (uncouplers of oxidative phosphorylation, anaerobiosis), which severely lower the ATP content in the cell, also inhibit basal as well as FC-induced proton extrusion (12, 16). A more accurate study of the relationship between the ATP content of the tissue and the activity of the proton-extruding system requires a precise measurement of the actual rate of proton extrusion. As a large fraction of the extruded protons seems to re-enter the cell by an anion and metabolite co-transport (4, 8, 15) and by diffusion, the titratable protons in the medium cannot be taken as repre-

senting the actual activity of the H^+-extruding system. The determination of the rate of K^+ uptake seems to provide a better evaluation of the activity of the system which extrudes protons on the basis of the hypothesis that the electrogenic proton extrusion is electrically or chemically coupled to the influx of K^+ (15). As the electrical component of the proton gradient built up by the active extrusion of protons is not affected by the electroneutral H^+-anion symport, K^+ influx (in the case of the electric coupling) accounts not only for the titratable H^+ in the medium but also for those re-entering with anions. Thus, the relationship between K^+ uptake and ATP level in the tissue has been studied.

The ATP level was changed *in vivo* by treating the maize-coleoptile segments with 2-deoxyglucose or Na-arsenate at various concentrations or by incubating the segments under limiting O_2 (3% O_2) or anaerobic conditions. All of these treatments decreased the ATP contents of the cell in a very short time; a new, almost constant (for about 1 h), level, peculiar to every treatment, was reached after 30 min (data not shown). Hence, the ATP levels of Figure 3 were measured after 30 min incubation and the determination of K^+ uptake was performed between 30 and 60 min treatment, when the ATP level remained virtually constant.

The data (Fig. 3) indicate that Na-arsenate, 2-deoxyglucose, and the decrease of O_2 availability determine a drop of ATP level in the controls, as well as in the FC-treated samples, and inhibit both basal and FC-stimulated K^+ uptake. Within each treatment, the inhibition of K^+ uptake roughly parallels the drop of ATP level both in the controls and in the FC-treated samples. The correlation between drop of ATP concentration and decrease of K^+ uptake is not quantitative. This is understandable as the mode of action of the inhibitors is quite different, and, due to ATP compartmentation, the changes of total ATP cannot be taken as expressing proportional changes of ATP in cytoplasm.

The effect of ATP-decreasing treatments on K^+ uptake seems more evident in FC-treated samples than in the controls; this might depend on the much higher flux rate in the FC-treated tissue.

FC stimulates K^+ uptake even when ATP level is very low, suggesting that the system activated by FC and responsible for stimulation of K^+ uptake has a high affinity for ATP. Only at extremely low ATP levels, as observed with 5 and 10 mM Na-arsenate, is the stimulation of K^+ uptake by FC suppressed.

The finding that a decrease of ATP level in the FC-treated tissue is still seen at Na-arsenate concentrations completely inhibiting FC-induced stimulation of K^+ uptake might be interpreted by assuming that, in this condition, FC still enhances ATP utilization by the pump, but it is no longer possible (because of either the ATP drop or of collateral consequences of Na-arsenate poisoning) to translate ATP hydrolysis into a stimulation of K^+ uptake.

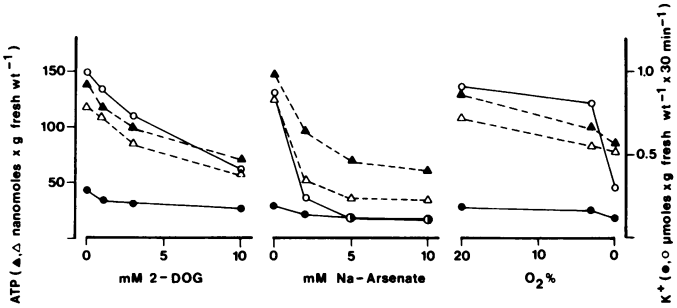

FIG. 3. Correlation between ATP levels and K^+ uptake in maize-coleoptile sections. Maize-coleoptile sections (50 mg/ml) were incubated in 1 mM KCl ± 20 μM FC and 2-deoxyglucose (2-DOG) or Na-arsenate or O_2 limiting for 30 min in the case of ATP level determinations and for 60 min in the case of K^+ uptake. ATP levels are expressed as nmol g^{-1} fresh weight. K^+ uptake is reported as μmol g^{-1} fresh weight 30 min^{-1} (between 30 and 60 min treatment). The experiments were repeated three times in triplicate; the values represent a typical experiment. (△, ○), FC; (▲, ●), controls.

DISCUSSION

The data reported here provide a further evidence supporting the hypothesis that the system, involved in the FC-promoted H^+/K^+ exchange, is an ATPase. In fact: (*a*) both the basal and the FC-induced K^+ uptake were severely inhibited by all treatments (Na-arsenate, 2-deoxyglucose, limiting O_2, anaerobiosis) lowering the ATP level of the tissue; (*b*) FC induced in the materials used here a rapid stimulation of Q_{O_2} and of K^+ uptake, with time courses quite similar to those described for H^+ extrusion and hyperpolarization of transmembrane potential difference (3, 14); (*c*) parallel to the stimulation of Q_{O_2} and K^+ uptake, FC determined a significant decrease of ATP level, as already reported for maize roots (5).

Inasmuch as FC does not appear to stimulate, at least in short-term experiments, any metabolic process requiring energy supply other than transport (9, 17), the FC-induced lowering of ATP content and the parallel stimulation of Q_{O_2} seem conveniently interpreted as depending on the primary effect of the toxin at the plasmalemma level.

The inhibition of FC-induced stimulation of O_2 uptake by NaN_3 and not by benzohydroxamic acid, and the lack of any uncoupling effect of FC in isolated mitochondria suggest that the extra O_2 uptake in the presence of FC is linked to ATP synthesis; assuming a P/O ratio of 3, the extra production of ATP would be about 50 μmol g^{-1} fresh weight h^{-1} in both materials. Under the same experimental conditions (1 mM KCl), the stimulation of K^+ uptake by FC is 1 to 2 μmol g^{-1} fresh weight h^{-1}. Therefore, calculating an ATP/K^+ ratio between 0.5 and 1 (11), there would be a very large excess of ATP consumption. The larger ATP utilization may depend on larger fluxes of ions stimulated by FC as a consequence of FC-induced hyperpolarization or by some uncoupling induced by FC at plasmalemma level.

Acknowledgment—We thank E. Marrè for valuable suggestions during the course of this work and for critical reading of this manuscript.

LITERATURE CITED

1. BALKE NE, TK HODGES 1979 Inhibition of adenosine triphosphatase activity of the plasma membrane fraction of oat roots by diethylstilbestrol. Plant Physiol 63: 48–52
2. BEFFAGNA N, S COCUCCI, E MARRÈ 1977 stimulating effect of fusicoccin on K-activated ATPase in plasmalemma preparations from higher plant tissues. Plant Sci Lett 8: 91–98
3. CLELAND RE, T LOMAX 1977 Hormonal control of H^+ excretion from oat cells. *In* E Marrè, O Ciferri, eds, Regulation of Cell Membrane Activities in Plants. Elsevier/North Holland Biomedical Press, Amsterdam, pp 161–171
4. COLOMBO R, MI DE MICHELIS, P LADO 1978 3-*O*-Methyl glucose uptake stimulation by auxin and by fusicoccin in plant materials and its relationships with proton extrusion. Planta 138: 249–256
5. GRONEWALD JW, JM CHEESEMAN, JB HANSON 1979 Comparison of the responses of corn root tissue to fusicoccin and washing. Plant Physiol 63: 255–259
6. HAROLD FM 1977 Membranes and energy transduction in bacteria. *In* DR Sanadi, ed, Current Topics in Bioenergetics, Vol 6. Academic Press, New York, pp 84–149
7. HODGES TK 1976 ATPases associated with membranes of plant cells. *In* U Lüttge, MG Pitman, eds, Encyclopedia of Plant Physiol, New Series Vol 2 Part A. Springer-Verlag, Heidelberg, pp 260–283
8. KOMOR E, M ROTTER, W TANNER 1977 A proton-cotransport system in higher plant: sucrose transport in *Ricinus communis*. Plant Sci Lett 9: 153–162
9. LADO P, F RASI-CALDOGNO, R COLOMBO 1977 Effect of cycloheximide on IAA- or FC-induced cell enlargement in pea internode segments. Plant Sci Lett 9: 93–101
10. LAMPRECHT W, I TRAUTSHOLD 1970 Adenosin-5′-triphosphatbestimmung mit hexokinase und glucose-6-phosphat dehydrogenase. *In* HU Bergmeyer, ed, Methoden der Enzymatischen Analyse, Band II. Verlag Chemie, Weinheim/Bergstr, pp 2024–2033
11. LÜTTGE U, MG PITMAN 1976 Transport and Energy. *In* U Lüttge and MG Pitman, eds, Encyclopedia of Plant Physiology, New Series Vol 2 Part A. Springer-Verlag, Heidelberg, pp 251–259

12. MARRÈ E, P LADO, F RASI-CALDOGNO, R COLOMBO 1973 Correlation between cell enlargement in pea internode segments and decrease in the pH of the medium of incubation. II. Effects of inhibitors of respiration, oxidative phosphorylation and protein synthesis. Plant Sci Lett 1: 185–192
13. MARRÈ E, P LADO, F RASI-CALDOGNO, R COLOMBO, MI DE MICHELIS 1974 Evidence for the coupling of proton extrusion to K^+ uptake in pea internode segments treated with fusicoccin or auxin. Plant Sci Lett 3: 365–379
14. MARRÈ E, P LADO, F RASI-CALDOGNO, R COLOMBO, M COCUCCI, MI DE MICHELIS 1975 Regulation of proton extrusion by plant hormones and cell elongation. Physiol Vég 13: 797–811
15. MARRÈ E 1979 Fusicoccin: a tool in plant physiology. Annu Rev Plant Physiol 30: 273–288
16. RASI-CALDOGNO F, R CERANA, MC PUGLIARELLO 1978 Effects of anaerobiosis on auxin- and fusicoccin-induced growth and ion transport. Experientia 34: 841–842
17. RASI-CALDOGNO F, P LADO, R COLOMBO, R CERANA, MC PUGLIARELLO 1978 Changes in cellular metabolism associated with the activation of H^+/K^+ exchange by auxin or fusicoccin in pea internode segments. Meeting of Federation of European Societies of Plant Physiology, Edinburgh, 221C
18. SCARBOROUGH GA 1976 The *Neurospora* plasmamembrane ATPase is an electrogenic pump. Proc Natl Acad Sci USA 73: 1485–1488
19. SLAYMAN CL, WS LONG, CYH LU 1973 The relationship between ATP and an electrogenic pump in the plasma membrane of *Neurosopra crassa*. J Membr Biol 14: 305–338
20. TOGNOLI L, N BEFFAGNA, P PESCI, E MARRÈ 1979 On the relationship between ATPase activity and fusicoccin binding capacity of crude and partially-purified microsomal preparations from maize coleoptiles. Plant Sci Lett 16: 1–14