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THE EFFECT OF THE UNCOUPLER CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE ON K^+ TRANSPORT, ATP LEVEL AND INTRACELLULAR pH OF *CHLORELLA FUSCA*

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1. Low concentrations of the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) induced net K^+ uptake by *Chlorella fusca*, optimal concentrations being 3 μM CCCP in the light and 1 μM CCCP in the dark. Higher concentrations increasingly stimulated K^+ release. 2. Measurements of the unidirectional K^+ fluxes showed that CCCP-induced net K^+ uptake in the light was mainly a consequence of an inhibition of efflux. In the dark, influx was slightly stimulated in addition. 3. In conditions of CCCP-induced net K^+ uptake, the ATP level was decreased by less than 10%. With higher CCCP concentrations it fell drastically. 4. By means of the 5,5-dimethyloxazolidine-2,4-dione distribution technique, an acidification of the cell interior on the addition of CCCP was found. 5. It is concluded that uncoupler-induced net K^+ uptake is due to an enhanced proton leakage into the cell across the plasmalemma. Intracellular acidification by this process stimulates ATP-dependent K^+/H^+ exchange which, in itself, is not affected at low uncoupler concentrations.

Introduction

A basic criterion for active transport is its dependence on metabolic energy. Inhibitors of energy metabolism, e.g. uncouplers, are expected to block such transport processes and to cause passive efflux of substances previously taken up actively. Measurements of K^+ content and membrane potential in *Chlorella* [1,2] showed that in this green alga, K^+ is accumulated against the gradient of the electrochemical potential. So it was surprising to find that the uncoupler CCCP induced net K^+ uptake, an evidently endergonic process [2] (Fig. 1). This effect is investigated in the present study.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DMO, 5,5-dimethyloxazolidine-2,4-dione; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N*-*N'*-bis(2-ethanesulfonic acid); TPP^+, tetraphenylphosphonium; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(2-(5-phenyloxazolyl)benzene.

CCCP acts by increasing the proton permeability of biomembranes. In the energy conserving membranes, this effect causes breakdown of the proton gradient [3] and hence also of ATP production. This leads to inhibition of the ATP-driven transport processes at the cell membrane. But in whole euraryotic cells, the uncoupler also affects the cell membrane directly. A different action of CCCP on cell membrane parameters and phosphorylation has been found with *Riccia* [4], the cell membrane potential and resistance being affected at much lower CCCP concentrations than phosphorylation. As in *C. fusca*, CCCP-induced net K^+ uptake was observed at relatively low concentrations of the uncoupler, in this case as well a direct effect on the plasmalemma was suspected. For this reason, the action of CCCP on K^+ transport has now been compared with its action on the ATP level of the cell.

Net K^+ uptake in *Chlorella* being due to K^+/H^+ exchange, is stimulated by acidification of the cell

interior [5]. As this fact offers an explanation for the stimulation of K^+ transport by CCCP, the intracellular pH of *C. fusca* in dependence on CCCP was also determined. The DMO distribution technique was used.

The experimental results in respect to ion fluxes, ATP level, and intracellular pH could explain the uncoupler-induced net K^+ uptake satisfactorily as a consequence of an enhanced proton leakage into the cell across the plasmalemma. Intracellular acidification by this process stimulates the ATP-dependent K^+/H^+ exchange. The K^+/H^+ pump itself is not affected at lower uncoupler concentrations.

Materials and Methods

Chemicals. ^{86}Rb was obtained by neutron activation of Rb_2CO_3. The chloride was made by addition of HCl to the carbonate and evaporation to dryness.

[2-^{14}C]DMO was bought from the Radiochemical Centre Amersham. CCCP, reagents and standard substances for the ATP assay were from Sigma, St. Louis. The luciferin-luciferase reagent was diluted according to Lundin and Thore [6].

Algae. Chlorella fusca (211-8b Göttingen) was cultivated and harvested as described [5]. The algae were suspended in a K^+-free medium consisting of 10^{-4} M $CaCl_2$ 5 mM Tris, and Pipes to give a final pH of 6.5, if not stated otherwise ('standard medium'). Cell density (vol% packed cells, determined by hematocrit) was 1.25%, corresponding to 2.5 g dry wt./l. The suspensions were aerated at 30°C and illuminated by fluorescent light (50 W/m^2) or kept in the dark. Before starting the experiments, the algae were preconditioned 2 h in the light, and for dark experiments an additional hour in the dark. After this time, K^+ release from the algae had ceased and the K^+ concentration in the supernatant was around 10^{-4} M and changed only slightly with time [5].

Determination of ion fluxes. Net K^+ movements were determined by following the K^+ concentration in the supernatant of the experimental suspension by atomic absorption spectrometry. The samples were obtained by centrifugation at 3000 $\times g$ for 1 min.

For the determination of Rb^+ uptake, 2 ml samples were filtered through Millipore filters (pore width 0.8 μm) and washed with 3 ml standard medium. The filters with the algae were transferred into counting vials containing 2 ml 1 M HCl, and the gamma radiation was determined by scintillation counting. The radioactivity of a 2 ml experimental suspension served as a standard.

For efflux measurements, the cells were suspended in $2 \cdot 10^{-4}$ M labelled RbCl for 2 h in the light. Then the algae were rapidly centrifuged at 3000 $\times g$ and washed once with standard medium. The loaded cells were then suspended in standard medium containing 10^{-4} M KCl (time zero of the experiment). The increase of the radioactivity of the supernatant was determined by measuring samples obtained by centrifugation at 3000 $\times g$ for 1 min.

Determination of ATP. In a 2.5 ml syringe, 1.12 ml algal suspension were rapidly mixed with 1.38 ml ice-cold extraction medium consisting of a 5 : 1 mixture of 1.5 N $HClO_4$ and 0.1 M EDTA. After 1 h storage at 0°C, the samples were neutralized with 0.7 ml of a solution containing 3 M KOH and 0.2 M Hepes. Precipitated $KClO_4$ and cell debris were centrifuged off and 2 ml of the supernatant were mixed with 5 ml 20 M Tris/H_2SO_4 buffer, pH 7.75. In this solution, ATP was determined by the firefly method with a Biolumat LB 9500, Berthold. As it has been found that the algal extracts contained quenchers, the data were corrected by means of an internal standard.

Measurement of the intracellular pH with DMO. [^{14}C]DMO was applied in a concentration of 10^{-5} M. Algal samples were obtained by filtration of 5 ml suspension as described above, only the washing was omitted. The cells were carefully sucked dry then the filter with the algae was extracted overnight with 2.5 ml 1 M HCl. After centrifugation, 2 ml of the supernatant were mixed with 10 ml scintillator cocktail (5 g PPO and 0.5 g POPOP per l of a 1 : 1 mixture of toluene and Triton X-100) and the radioactivity was determined by liquid scintillation counting. For the determination of total radioactivity, 0.5 ml of the experimental suspension were extracted with 2 ml 1 M HCl. After centrifugation, 2 ml of the supernatant were counted in the same way.

It was found that 0.3 ± 0.1% of the DMO were absorbed by the filter. Internal cell water was assumed to be 60% of the total cell volume [1], the remaining 40% being filled with DMO of the concentration of the external medium. For the calculation of the intracellular pH the equation used was:

$$\frac{c_e}{c_i} = \frac{(100 - 0.3 - X)\,0.6D}{(1000 - 0.6D)\,X} = \frac{1 + 10^{(pH_e - 6.13)}}{1 + 10^{(pH_i - 6.13)}}$$

where c_e, c_i = external and intracellular concentration of DMO; pH_e, pH_i = external and intracellular pH; X = % DMO in the algae, and D = cell density in ml cells/l suspension.

Results

Ion movements

Low concentrations of CCCP induced net K^+ uptake in *C. fusca* after a short time of K^+ release (Fig. 1). The CCCP concentrations for maximum K^+ uptake were 3 μM in the light and 1 μM in the dark, i.e. below the concentrations of 10–50 μM commonly used for uncoupling algal respiration [7]. The uncoupling concentrations caused a permanent K^+ loss from the cells.

For measurements of K^+ influx and efflux ^{86}Rb was used as a label. The use of Rb^+ in K^+ transport studies with plants is well documented, also in the case of *Chlorella* [8–10]. Rb^+ influx in the light proved to be almost independent of CCCP up to a concentration of 10 μM. With 50 μM CCCP, however, the influx was totally blocked (Fig. 2a). In the dark, stimulation of influx was found with 1 μM CCCP and

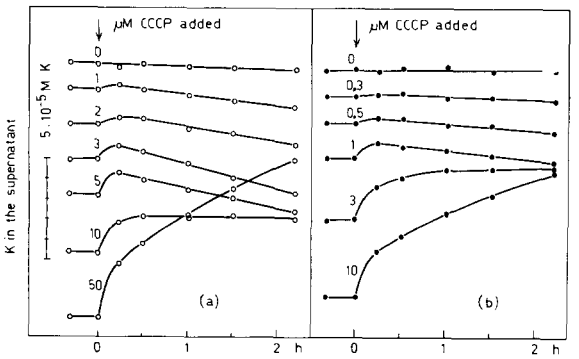

Fig. 1. Effect of CCCP on net K^+ movements in the light (a) and in the dark (b). Before addition of CCCP, the curves are identical with K^+ concentrations of $0.85 \cdot 10^{-4}$ M in (a) and $1.35 \cdot 10^{-4}$ M in (b). For better comparison, the curves are drawn separately.

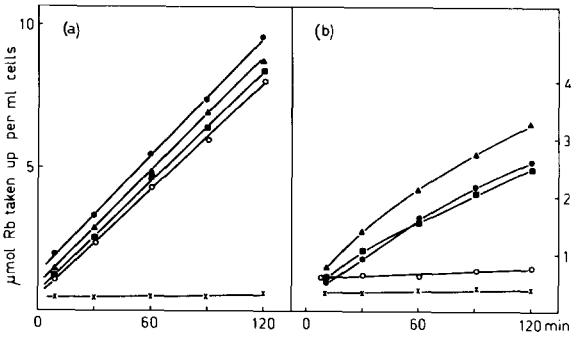

Fig. 2. Effect of CCCP on uptake of Rb^+ in the light (a) and in the dark (b). (•) Control; (▲) 1 μM CCCP; (■) 3 μM CCCP; (○) 10 μM CCCP; (X) 50 μM CCCP. At time zero, K^+ concentration in the supernatant was $0.70 \cdot 10^{-4}$ M in (a) and $0.95 \cdot 10^{-4}$ M in (b). Rb^+ concentration was 10^{-4} M.

almost total inhibition was reached with 10 μM CCCP (Fig. 2b). Rb^+ efflux from loaded cells in the light was considerably reduced in presence of 1 μM and 3 μM CCCP (Fig. 3a). In the dark, only a small inhibition was found with 1 μM CCCP (Fig. 3b). So CCCP-induced net K^+ uptake in the light is due to an inhibition of efflux; in the dark it is mainly a consequence of stimulation of influx.

ATP level

The action of CCCP on the ATP level of *C. fusca* is shown in Fig. 4. Phosphorylation is affected more strongly in the dark than in the light. Up to 3 μM CCCP in the light and up to 1 μM CCCP in the dark, the ATP levels are depressed by less than 10% of the

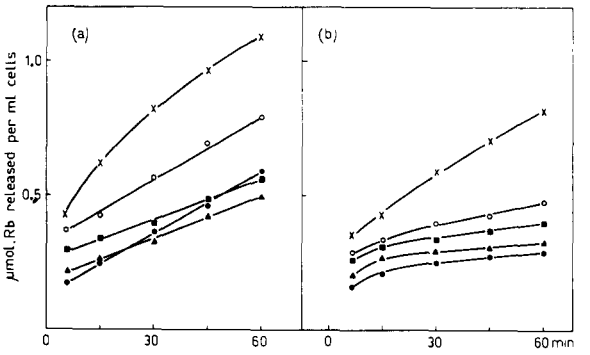

Fig. 3. Effect of CCCP on efflux of Rb^+ in the light (a) and in the dark (b). Symbols as in Fig. 2.

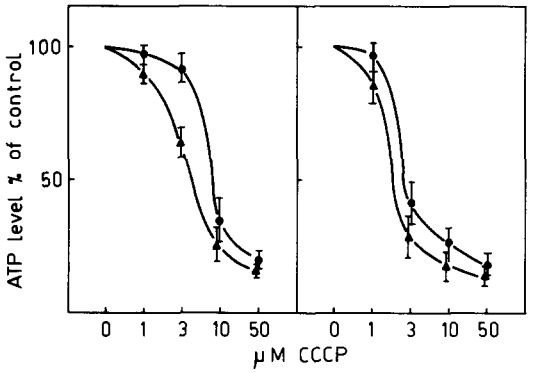

Fig. 4. Action of CCCP on the ATP level of *C. fusca* in the light (a) and in the dark (b). Mean of three experiments. (•) 10 min; (▲) 45 min after addition of the uncoupler. The absolute ATP levels of the control ranged between 0.7–1.2 μmol ATP/ml cells.

control. At higher concentrations evidently uncoupling occurred. Some recovery of phosphorylation after prolonged CCCP action could be observed, the ATP levels being higher after 45 min than after 10 min of CCCP action.

DMO uptake

Constant distribution of DMO was reached after 5–8 min. In most experiments, this distribution remained fairly constant with time, only in a few cases an increase of DMO uptake corresponding to an increase of the intracellular pH by 0.1–0.2 units/h was observed. CCCP depressed DMO uptake more strongly in the light than in the dark (Fig. 5). At an external pH of 6.5, the internal acidification by 50 μM CCCP was 0.25 units in the light and 0.15 units in the dark (Fig. 6a). As in the dark, internal pH of the controls was always by 0.1–0.15 units more acidic than in the light. The internal pH in presence of 50

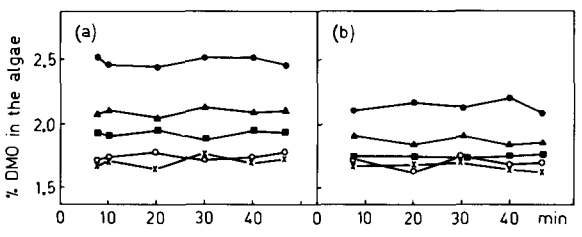

Fig. 5. Effect of CCCP on DMO uptake. Symbols as in Fig. 2.

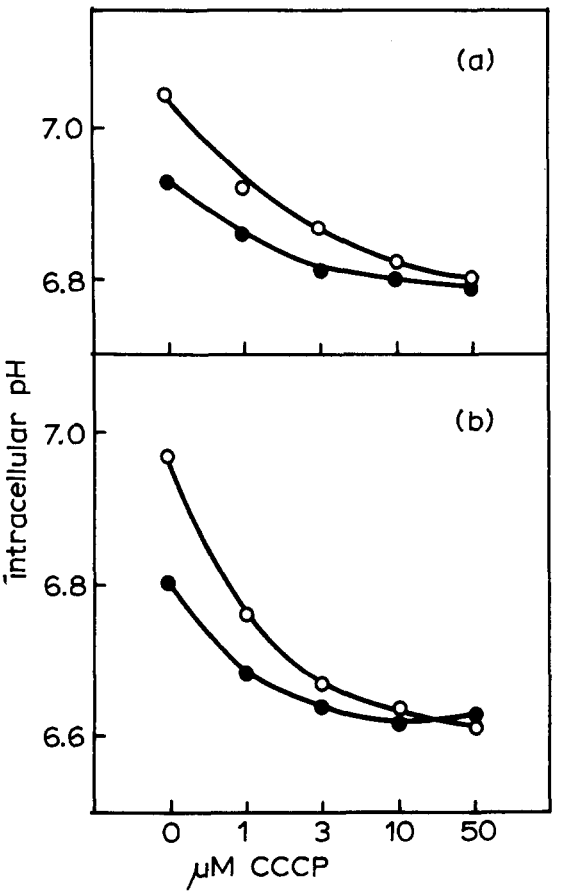

Fig. 6. Dependence of intracellular pH on CCCP concentration at pH 6.5 (a) and pH 6.0 (b). Light (○); dark (•).

μM CCCP was about the same under both conditions (Fig. 6). At an external pH of 6.5, DMO uptake is only around 2% of total DMO. For better accuracy, experiments were also carried out at pH 6.0. At this pH, the internal acidification by CCCP is more pronounced, with 0.35 units in the light and 0.20 units in the dark (Fig. 6b).

Discussion

It is well established that plant cells maintain constant internal pH near neutrality by means of active extrusion of protons [11]. Proton fluxes cannot be measured directly. The use of tritium as a label is not possible because of the fast proton exchange in water and the high water permeability of biomembranes.

pH effects in the external medium, however, are often obscured by pH changes arising from other metabolic reactions. Thus, our knowledge of proton transport rests mainly on indirect methods, e.g. measurements of transport processes and membrane properties in dependence of external and internal pH. In this way, in *C. fusca* the influence of salts of permeant acids and bases has indicated the existence of a membrane-bound K^+/H^+ exchange system that acts as a pH-stat of the cell. Internal acidification leads to extrusion of protons and uptake of K^+, alkalization to proton uptake and K^+ efflux [5]. Measurements of the membrane potential by means of the lipophilic cation TPP^+ indicated that this K^+/H^+ exchange operates independently of the membrane potential [2].

C. fusca has a membrane potential around 100 mV (inside negative). A potential of this magnitude can accumulate cations about 50-fold. Thus at pH 6.5 and an external K^+ concentration of 0.1 mM, an internal pH of 5 and an internal K^+ concentration of 5 mM would be in thermodynamic equilibrium. The actual values of pH 7 and 170 mM [2] are evidently a consequence of active proton extrusion and K^+ uptake. As CCCP is a protonophore, it should cause a proton leak in the cell membrane and the internal pH should fall. This has actually been found (Fig. 6) in accordance with other studies with *Chlorella vulgaris* and FCCP [12]. Compensation of the enhanced proton influx by K^+/H^+ exchange needs metabolic energy as both H^+ extrusion and K^+ uptake are endergonic in the conditions given.

The ATP measurements show that this energy actually can be supplied: in conditions of CCCP-induced K^+ uptake, the ATP level is nearly unimpaired. The small K^+ loss which precedes net K^+ uptake may be due to a transient depolarization by uncoupler-mediated electrogenic proton influx. Evidently, the carrier system needs some time to deal with the changed conditions. A permanent K^+ loss from the cells is only observed with CCCP concentrations that also depress the ATP level drastically (Figs. 1 and 4).

In *C. fusca*, as in most cells, intracellular K^+ exchanges readily with external K^+ [13,14]. In the light, under conditions of CCCP-induced net K^+ uptake, only the efflux is decreased (Figs. 2a and 3a). This suggests that the turnover of the cation exchange system remained constant, only a greater part of the K^+ influx now being balanced by proton efflux. Probably K^+ and H^+ compete for an internal transport site the affinity of which depends on the internal pH. In the dark, beside this effect also the turnover of cation exchange seems slightly increased.

Kinetically in *C. fusca* two uptake systems for K^+ can be distinguished. In the low concentration range up to 1 mM, mechanism I is operating which has a high affinity to K^+ (K_M for $Rb^+ = (3.8–18.2) \cdot 10^{-5}$ M according to Kannan [9], and $7 \cdot 10^{-5}$ M according to Solt et al. [15]). Mechanism II of lower affinity (K_M for $Rb^+ = 6.45–12.38$ mM according to Kannan [9], and 3 mM according to Solt et al. [15]) probably mediates only K^+/K^+ exchange [10]. These two mechanisms may not represent two different uptake systems but rather two states of the same system. In his studies with *Chlorella*, Barber observed a 70% inhibition of both influx [13] and efflux [14] in presence of 10 μM CCCP. As these measurements were made in a nutrient medium containing 6.5 mM K^+, i.e. in the range of mechanism II, they cannot be compared directly with the present results that refer exclusively to mechanism I, which may have a different sensitivity to CCCP.

Evidently, the cell membranes of *Chlorella* and *Riccia* [4] are more sensitive to uncouplers than the energy-conserving membranes of their mitochondria and chloroplasts. The reason for this difference is surely not the more exposed localization of the cell membrane as uncouplers diffuse rapidly. Neither can differences of solubility and mobility of the uncoupler within the membrane explain the great discrepancies in sensitivity. The main reason rather seems to be the far higher proton turnover in the energy-conserving membranes.

Respiration in *Chlorella* is in the range of 1 μmol O_2/ml algae per min [16]. The proton flux in the mitochondria can be assumed to be at least 12-fold higher [3,17]. With a 1 : 1 ratio between K^+ uptake and H^+ extrusion, the H^+ flux across the plasmalemma will not exceed K^+ influx, which is $4 \cdot 10^{-8}$ mol $Rb^+ + K^+$/ml cells per min in the dark (Fig. 2b). This value is an upper limit, as a substantial part of K^+ influx is due to K^+/K^+ exchange. If the areas of the inner mitochondrial membrane and of the plasmalemma were assumed to be equal, the density of proton fluxes would be 300-times higher in the inner mitochondrial

membrane. As the area of the latter surely does not exceed the area of the plasmalemma by two orders of magnitude, the higher proton flux density in the mitochondrial membrane can be taken for granted. Similarly also a far higher proton turnover in the thylakoid membrane compared to the cell membrane can be deduced. The lesser sensitivity of the ATP level to CCCP in the light (Fig. 4) may indicate that the proton flux density is even higher in the thylakoid membrane than in the inner mitochondrial membrane.

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