Biochimica et Biophysica Acta, 464 (1977) 179—187

BBA 77564

A STUDY OF THE PRIMARY EFFECT OF THE UNCOUPLER CARBONYL CYANIDE *m*-CHLOROPHENYLHYDRAZONE ON MEMBRANE POTENTIAL AND CONDUCTANCE IN *RICCIA FLUITANS*

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(Received April 26th, 1976)
(Revised manuscript received August 13th, 1976)

Summary

1. In the presence of 10^{-5} to 10^{-8} M carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) the membrane potential of thallus cells of the aquatic liverwort *Riccia fluitans* responds to changes of the external pH between 5.5 and 8.3. This occurs in the light and dark, and also if respiration is abolished by addition of 10^{-4} M KCN and 10^{-5} M salicyl-hydroxamic acid.

2. The ATP-level of the thallus is reduced, independently of the external pH, by $\geqslant 10^{-6}$ M CCCP to 30—40% of the control level of about 1.1 nmol ATP per mg dryweight within 5 min.

3. Upon addition of 10^{-4} M CCCP at 20°C the ATP-level declines exponentially with a half time of about 20—30 s, whereas the membrane potential declines exponentially with a half time of about 2—3 s.

4. At pH 7.2 the electrical membrane conductance yields a sigmoid curve as a function of the logarithm of the CCCP concentration between 10^{-8} and $3 \cdot 10^{-6}$ M. On the other hand, at $3 \cdot 10^{-7}$ M CCCP the g_m (electrical slope conductance, $\mu S \cdot cm^{-2} = 10^{-6} \cdot \Omega^{-1} \cdot cm^{-2}$) versus pH-curve displays an optimum between pH 6.5 and 7.5.

5. We conclude that CCCP acts upon membrane potential and conductance in *Riccia* predominantly by inducing a passive proton permeability of the cell membrane, i.e. CCCP raises the permeability ratio, P_H/P_K, more than 100-fold above its control level of about 10.

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCMU, 3-(3,4-dichlorophenyl) 1,1-dimethylurea.

Introduction

From experiments with artificial membranes [1—4] as well as mitochondria [5,6] we know that certain weak acids like carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), well-known as uncoupling agent of the oxidative and photosynthetic phosphorylation, increase the proton permeability of membranes. Generally this function is explained by a carrier-shuttle mechanism which seems to be due to the good lipid solubility of the CCCP-anion. One electrophysiologically measurable effect of CCCP upon the plasmalemma is the depolarization of its potential. Mostly this is explained by the uncoupling of the ATP synthesis in the organells. The decline in cellular ATP concentration of the cell then affects the electrogenic ion pump which is generally assumed to be located at the plasmalemma and fueled by ATP [7,8]. It is the purpose of this paper to show that besides this known effect of CCCP on the membrane potential an immediate effect of CCCP upon the proton permeability of the plasmalemma exists. The general electrical and ion transport properties of the *Riccia* cell membrane have been described recently [9,10].

Material and Methods

Material and medium

Thalli of *Riccia fluitans* were grown in a greenhouse in natural pond water under a 12/12 h light/dark period. For measurements the plants were preincubated 24 h in a test solution, usually containing 0.1—1 mM K^+, 2—2.9 mM Na^+, 1 mM Cl^- and buffered with 2 mM sodium phosphate. The pH was varied from 8.3 to 4.7. The agents CCCP, salicyl-hydroxamic acid and 3-(3,4-dichlorophenyl)1,1-dimethylurea (DCMU) were predissolved in 1—2 ml of ethanol or methanol and stirred into the test solution carefully. Aqueous solutions of CCCP decay quite rapidly [3], therefore they were always freshly prepared just before the experiment.

Electrical measurements

For voltage- and resistance-measurements the glass-microelectrode technique has been applied [10,11]. Voltages between the vacuole and the test medium have been measured on green thallus cells or on the colorless, cylindrical rhizoid cells, and were recorded by a high impedance amplifier (Keithley 610 C) to a pen chart recorder.

Resistance measurements were carried out on the rhizoid cells by injecting the test current with a separate electrode at different distances from the voltage electrode [11].

Measurements of intracellular ATP

Thallus samples of 5—10 mg dryweight were killed in light petroleum and cooled to —79° C with carbon dioxide. After freeze-drying for 48 h the samples were weighed and ATP was extracted for 30 min at 0° C with 6% $HClO_4$. The extracts were neutralized and centrifuged for 15 min at 7000 rev./min. The supernatant was diluted and assayed for ATP by means of luciferin/luciferase firefly extract (Sigma) [12]. The bioluminescence was measured using a bio-

luminescence equipment (XP-2000, Skan Ag, Basel), or a liquid-scintillation-counter (Berthold-Frieseke 2000).

Results

The effect of external pH and CCCP on the membrane potential

In the test solutions without CCCP, only a small effect of the external pH on E_m (membrane (vacuolar) potential) in the light or dark could be detected. In the pH range from 5.5 to 8.3 there is no significant response of E_m at all; from pH 5.5 to 4.7 a mean depolarization of about 10—15 mV was observed. Fig. 1 shows that CCCP drastically enlarges the effect of the external pH on E_m; its effect on E_m in the dark seems to be greater than in the light. Solutions containing both, 10^{-4} KCN and 10^{-5} M salicyl-hydroxamic acid reduce E_m as shown by Figs. 1 and 2. E_m values at 4°C are similarly low (Felle, H., unpublished). E_m can be further reduced by addition of $\geqslant 10^{-7}$ M CCCP in the range of pH 7 to 5 (Fig. 2).

The effect of external pH and CCCP on the membrane conductance

Fig. 3 shows a peak of the electrical membrane conductance of about 40 $\mu S/cm^2$ at pH 7 in the interval of pH 8.3 to 4.7, if $3 \cdot 10^{-7}$ M CCCP is present. Without CCCP a sigmoid curve is obtained levelling off with g_m (electrical slope conductance, $\mu S \cdot cm^{-1} = 10^{-6} \cdot \Omega^{-1} \cdot cm^{-2}$) = 6 $\mu S/cm^2$ at pH 8.3 and g_m = 55 $\mu S/cm^2$ at pH 4.7. A similar curve is given by the g_m versus CCCP-function, if the external pH is 7.2 (Fig. 4).

ATP-level and -turnover

CCCP-concentrations below $3 \cdot 10^{-7}$ M do not measurably influence the ATP-level in *Riccia* thallus cells, but at higher concentrations of CCCP a significant decrease of the ATP-level was detected depending upon the time of incubation (Fig. 5). Small effects of CCCP on the ATP-level are difficult to assess, because

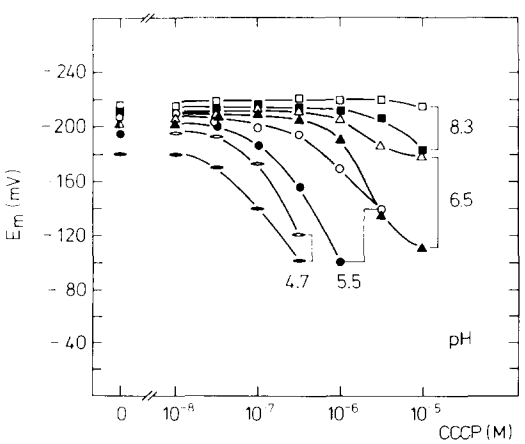

Fig. 1. Membrane potential (E_m) of *Riccia fluitans* in the light (open symbols) and dark (closed symbols) at different concentrations of CCCP and external pH as indicated. Mean values from 5 experiments each.

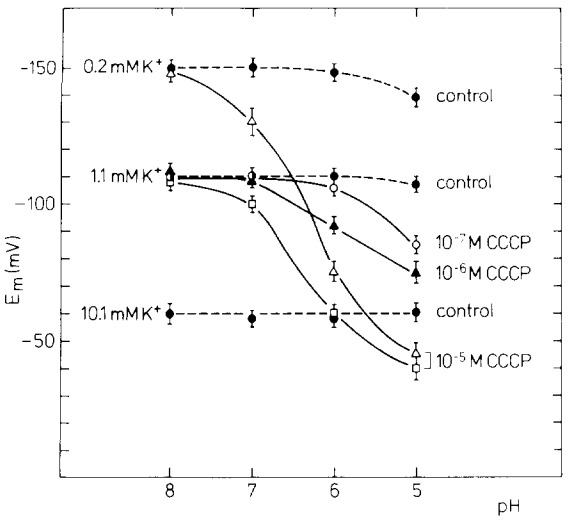

Fig. 2. Membrane potential (± S.E.M.) of *R. fluitans* exposed to light, 10^{-4} M KCN, 10^{-5} M DCMU, and 10^{-5} M salicyl-hydroxamic acid as a function of the external pH. Different concentrations of CCCP and K^+ as indicated. Controls (no CCCP) are given for 0.2, 1.1, and 10.1 mM K^+ by the dotted curves.

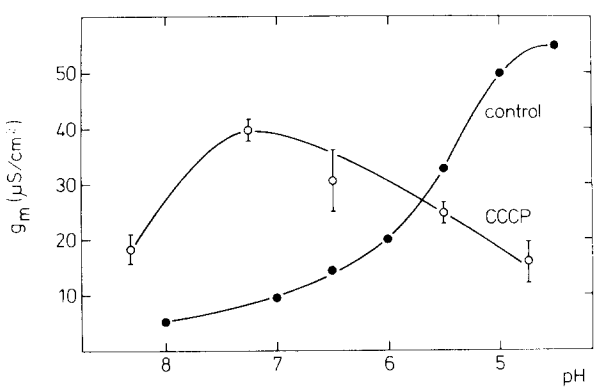

Fig. 3. Electrical membrane conductance (± S.E.M.) of *R. fluitans* in the light as a function of the external pH at $3 \cdot 10^{-7}$ M CCCP and 0.1 mM K^+.

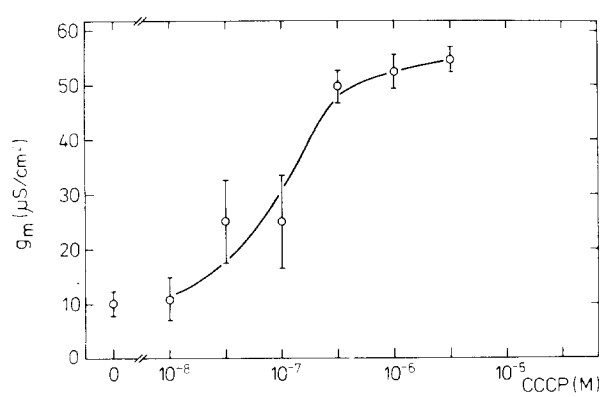

Fig. 4. Membrane conductance (± S.E.M.) as a function of different CCCP-concentrations at pH 7.2 and 0.1 mM K^+.

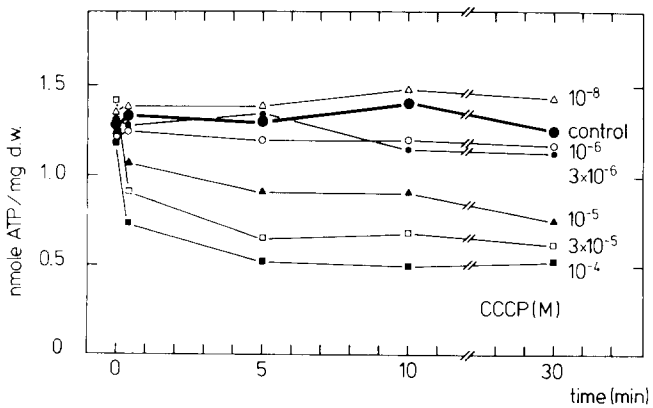

Fig. 5. ATP-level of thalli of *R. fluitans* after addition of CCCP at t = 0 (concentrations as indicated). S.E.M. was ±0.08 nmol ATP/mg dryweight from 3 experiments. K^+ was 0.1 mM.

the ATP-level tended to oscillate. If the cells were incubated for 30 min in test solutions of different CCCP-concentration and pH, the ATP-level dropped as shown in Fig. 6. In general, even incubation with CCCP for several hours did not reduce the ATP-level by more than 70%.

Table I gives information on the ATP-level in the presence of other inhibitors, that is KCN, salicyl-hydroxamic acid and DCMU and combinations of them in the light and dark. It should be noted that the ATP-level after 15 min of incubation in the dark with KCN, and salicyl-hydroxamic acid does not differ significantly from the ATP-level measured after incubation with KCN, CCCP, and salicyl-hydroxamic acid.

Kinetics of membrane depolarization and decrease in ATP upon addition of CCCP

At high (10^{-4} M) CCCP and low pH a two-phased depolarization of E_m occurs (Fig. 7). An initial phase with a half-time of only 2—3 s is followed by a tempo-

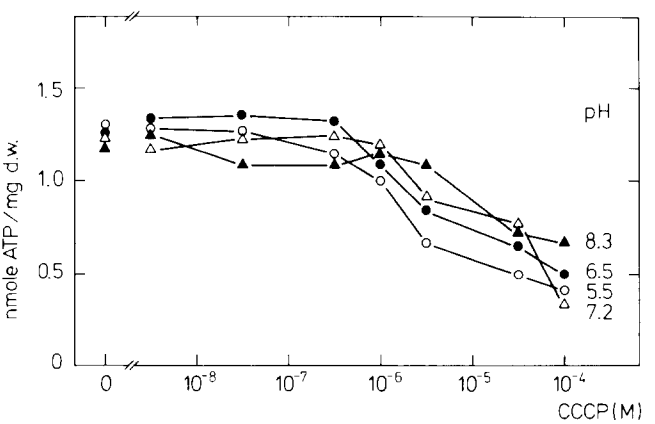

Fig. 6. ATP-level at 30 min after addition of CCCP at the indicated pH values. S.E.M. was ±0.09 nmol ATP/mg dryweight from 4 experiments. K^+ was 0.1 mM.

TABLE 1

ATP-LEVELS (± S.E.M.) IN THALLI OF *RICCIA FLUITANS* EXPOSED FOR 15 MIN TO DIFFERENT INHIBITORS IN ARTIFICIAL POND WATER OF pH 6; K^+ WAS 1.1 mM.

Inhibitor	Molarity (M)	nmol ATP/mg dryweight	
		Light	Dark
Control	—	1.267 ± 0.027	1.296 ± 0.038
CCCP	10^{-5}	1.140 ± 0.073	1.049 ± 0.094
KCN	10^{-4}	1.156 ± 0.013	1.142 ± 0.058
Salicyl-hydroxamic acid	10^{-5}	1.256 ± 0.060	1.214 ± 0.118
KCN, salicyl-hydroxamic acid	10^{-4}, 10^{-5}	1.212 ± 0.027	0.780 ± 0.009
KCN, CCCP, salicyl-hydroxamic acid	10^{-4}, 10^{-5}, 10^{-5}	0.731 ± 0.023	0.762 ± 0.015
KCN, DCMU, salicyl-hydroxamic acid	10^{-4}, 10^{-5}, 10^{-5}	0.733 ± 0.036	0.759 ± 0.031

rary quasi-stationary membrane potential and later on (not shown in Fig. 7) by a further much slower depolarization in the order of min. On the other hand, g_m increases in the initial phase of rapid depolarization but remains constant or decreases slightly during the following phase.

The ATP-level, on the other hand, drops at a clearly smaller rate. Values between 20 and 30 s under the same conditions have been found (Fig. 7).

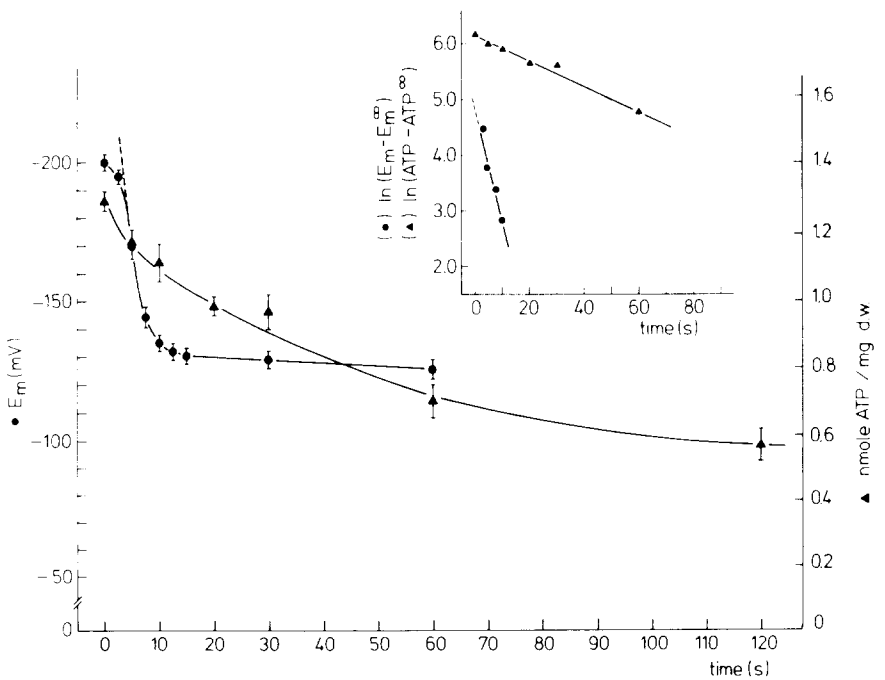

Fig. 7. Time-course of ATP-decay (▲) and membrane depolarisation (•) after addition of 10^{-4} M CCCP at $t = 0$. Inset shows the slopes of the natural logarithm of the difference between the measured values of E_m and ATP, and the extrapolated asymptotes, E_m^∞ and ATP^∞, respectively.

Discussion

Depolarization of the membrane potential and changes in proton permeability

Any evidence for an immediate effect of the uncoupler CCCP upon plasmalemma properties requires that it could be separated from the well-known action of this uncoupler upon phosphorylation. The results presented in Figs. 5 and 6 show that CCCP at concentrations $\gtrsim 3 \cdot 10^{-6}$ M observably reduces the total ATP content of *Riccia* thallus cells. We have previously shown that the putative ATP-fueled electrogenic proton export pump operates also in the *Riccia* plasmalemma, in fact, governs its membrane potential under most conditions [10].

Hence in the presence of $\gtrsim 10^{-6}$ M CCCP the observed decrease in membrane potential (Figs. 1 and 2) could easily be atributed to the reduced ATP-level and thus conceal any immediate effect of CCCP upon the plasmalemma. However, the result of Fig. 7 rules out a close correlation between E_m and ATP-level. After addition of 10^{-4} M CCCP E_m drops at a much faster rate than the ATP-level. This fast depolarization of the plasmalemma could well indicate that CCCP immediately acts upon the electrical properties of this membrane. In the following it is argued that CCCP acts as a proton carrier modifying the electrical properties of the plasmalemma of *Riccia*.

This conclusion will be inferred from the effect upon membrane potential and conductance of CCCP at concentrations $< 10^{-6}$ M which are evidently too low to reduce the measured ATP-level (Figs. 5 and 6).

In a previous paper we have concluded that the passive proton permeability of the plasmalemma is low so that in the light the membrane potential is governed by the electrogenic pump, and its small response to changes in external pH is mediated by the electromotive force of this proton pump rather than by H^+ diffusion through passive channels [10]. Therefore, the result in Figs. 1 and 2 that in the presence of CCCP the membrane becomes highly sensitive to changes in external pH seems plausible: CCCP creates a de novo proton permeability.

The results of Table I fairly indicate that neither KCN nor salicyl-hydroxamic acid have a notable influence on the ATP-level. Evidently, respiration in *Riccia*, like in *Neurospora* [13], has a compensatory cyanide-insensitive bypass which can be inhibited by salicyl-hydroxamic acid. Photosynthetic phosphorylation, however, to a certain extent compensates for the absence of the oxidative phosphorylation.

The result that the ATP-level measured after joint addition of KCN and salicyl-hydroxamic acid in the dark does not differ significantly from the ATP-level measured after joint addition of the three inhibitors, KCN, salicyl-hydroxamic acid and CCCP in the light and dark, strongly supports our idea that the depolarization of the plasmalemma in the presence of these inhibitors cannot be due to the inhibition of the proton pump via ATP, but must be attributed to a passive proton transport induced by CCCP.

The stationary level of E_m in the presence of KCN and salicyl-hydroxamic acid given in Fig. 2 resembles the diffusional component of E_m which has been derived from the response of E_m to external K^+ and H^+ in previous work [10] and from experiments carried out at 4°C where the electrogenic pump seems

TABLE II

VALUES (± S.E.M.) OF THE PERMEABILITY RATIO, P_H/P_K, OF THE *RICCIA* CELL MEMBRANE

The values are calculated from the data of Fig. 2 using the Goldman equation (Eqn. 1 in the text). The control value is about 10 in the pH range from 4.5 to 6.5.

pH	CCCP (M)		
	10^{-7}	10^{-6}	10^{-5}
7	—	690 ± 250	5340 ± 1050
6	210 ± 60	1100 ± 100	6510 ± 520
5	180 ± 20	470 ± 180	1620 ± 160

to stall (Felle, H., unpublished). Explicitly, using the Goldman constant field equation for E_m, i.e.

$$E_m = \frac{RT}{F} \ln \frac{P_K \cdot K_i^+ + P_H \cdot H_i^+ + P_{Na} \cdot Na^+{}_i}{P_K \cdot K_o^+ + P_H \cdot H_o^+ + P_{Na} \cdot Na_o^+} \quad (1)$$

where R, T, and F have their usual meanings, and the subscripts i and o refer to the vacuolar and external ion concentrations, respectively, a relative permeability ratio, P_H/P_K of about 10 has been derived for a medium containing no CCCP [10]. Similarly, from the data of Fig. 2 such relative permeabilities for different values of pH and concentrations of CCCP have been estimated and laid out in Table II. The indicated increase of the P_H/P_K ratio is attributed to an increase in the proton permeability, P_H, rather than a decrease in P_K. Addition of KCN, for instance, increases K_o^+; this could account for an increase of P_K only. Moreover, in Fig. 2 comparison of the curves of 0.2 and 1.1 mM K^+ and 10^{-5} M CCCP each, shows that E_m essentially responds to changes in external pH rather than K^+.

The membrane conductance as a function of CCCP and pH

It is most probable that CCCP acts as a proton carrier in the *Riccia* plasmalemma and tonoplast, that is, constitutes a proton conductance, g_H, within these cell membranes. Evidence for this, firstly, is offered by the comparison of Figs. 1 and 4: g_m increases at CCCP-concentrations which do not reduce E_m, hence no voltage-dependent change of g_m can be involved (cf. Ref. 10).

Secondly, this carrier mechanism can be inferred from the optimum curve for g_m given in Fig. 3. A passive proton influx which is carried by the CCCP molecules and driven by E_m should increase g_m, because it constitutes an electrical current. Explicitly, and increase in the membrane current density, ΔJ_m, increases g_m according to Ohm's law, that is, $\Delta g_m = \Delta J_m/E_m$. The optimum curve of g_m in Fig. 3 suggests that the low H^+ concentration at pH 8.3 limits g_H, hence g_m; whereas at pH 4.7 g_H is limited by the low dissociation of CCCP (pK = 5.95). Such optimum curves have been reported for the electrical conductance of CCCP-treated artificial membranes [3]. Electroneutrality requires that another ion flux must balance the proton influx. Since E_K is always more positive than E_m, a K^+ efflux could provide this counter-current.

Acknowledgement

This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

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