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Correlation of Adenosine Triphosphate Levels in *Chara corallina* with the Activity of the Electrogenic Pump[1]

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ABSTRACT

The effect of a number of inhibitors on the ATP level in single cells of *Chara corallina* has been measured using the luciferin-luciferase assay. The uncouplers of phosphorylation, carbonyl cyanide *m*-chlorophenyl hydrazone and 2,4-dinitrophenol, and the ATPase inhibitors, dicyclohexylcarbodiimide and diethylstilbestrol, all caused a marked reduction of the ATP level. These inhibitors also produced a large increase in the membrane resistance and a depolarization of the membrane potential to the diffusion potential. This is consistent with the plasmalemma containing an ATP-dependent electrogenic pump that provides the primary conductance through the membrane.

Ethyl-3-(3-dimethylaminopropyl)carbodiimide, which depolarizes the membrane potential, has no effect on the ATP level and does not increase the membrane resistance. This inhibitor apparently does not enter the cell but may act by affecting the permeability of the membrane. Neither darkness nor 3-(3,4-dichlorophenyl)-1,1-dimethylurea lowers the ATP level and, while neither has much effect on the membrane potential, both cause a similar increase in resistance in comparison with the control in the light. The weak acid, 5,5-dimethyloxazolidine-2,4-dione, and the weak base, NH_3, do not affect the ATP level significantly but have effects on the electrogenic pump that are consistent with their postulated effects on the cytoplasmic pH, if H^+ is the substrate for the pump.

An understanding of active ion transport in plants requires knowledge of the energy supply available for transport. Often this aspect of the transport system is characterized by the effect of various metabolic inhibitors on transport. In using these inhibitors, care must be taken to avoid preconceived notions about the energy supply in whole cells based on extrapolation from studies on isolated chloroplasts or mitochondria. As an example of the problems that may arise, it has sometimes been implied that light-stimulated transport is due to an increase in ATP concentration in the light (11), yet seldom has the ATP level been measured under similar conditions. When the levels of ATP in the light and dark were measured, they were found to be the same: Jeanjean (3) in *Chlorella*, Penth and Weigl (13) in *Chara foetida*, Spanswick and Miller (21) in *Nitella translucens*, and Lüttge and Ball (10) in greening barley leaves. Thus, knowledge of the change in the energy level is essential before changes in the energy level can be used to explain changes in ion transport rates.

In *Chara corallina*, the membrane potential is maintained in a hyperpolarized state, compared with the diffusion potential, by an electrogenic ion pump in the plasmalemma that, at an external pH of 6, probably carries H^+ and is the major conducting element in the membrane (5). If this electrogenic pump is an ATPase, reduction of the ATP level should depolarize the membrane potential to a value nearer the diffusion potential and decrease the membrane conductance. Measurements of the membrane potential and resistance under a number of inhibited conditions have already been reported for *C. corallina* (5). This paper reports measurements of ATP levels under conditions similar to those used for the electrical measurements, to examine the relationship between the ATP levels and the electrical properties of the membrane at an external pH of 6. A correlation between these properties would indicate that the electrogenic pump observed at pH 6 is probably an ATPase as is the case in *Neurospora crassa* (18). Some of these data have been presented in preliminary form (4).

MATERIALS AND METHODS

The ATP concentration in the cells was measured using the luciferin-luciferase assay (23). The basic method was that of Schram (16) using a scintillation counter with only one photomultiplier tube (noncoincident mode) to measure the light production. The details of this assay were worked out in this laboratory by A. G. Miller. The luciferin-luciferase was from a crude extract of 50 mg of firefly lanterns premixed with buffer and desiccated (Sigma Chemical Co.). This enzyme was prepared for use by adding 5.0 ml of water, storing on ice overnight to reduce the background, and filtering prior to use.

The assay buffer contained 25 mM Hepes, 25 mM $MgSO_4$, and was adjusted to pH 7.5 using 1 N NaOH. The ATP standard was made to 1.0 mM in this assay buffer and stored frozen until needed. Just before use, the ATP was thawed and diluted 1,000-fold with assay buffer. It was then kept on ice throughout the experiment.

The cells used were internodal cells of *C. corallina* Klein ex Willd., em. R.D.W. (= *C. australis* R.Br.). They were cultured (5) in the greenhouse under natural light supplemented with fluorescent lamps to provide a day length of 16 h. The cells were cut from the plant the evening before the experiment and the lengths and diameters measured. These cells were left overnight in the dark at 25 C in APW6[3] + 0.4 mM KCl (20). Ninety min prior to the start of an experiment the cells were put in fresh solution in a water bath at 25 C and illuminated with an incandescent lamp at a photon flux density (400–700 nm) that was typically 55 to 65 $\mu E/m^2 \cdot s$ (measured with a LI-COR quantum radiometer). At the start of the experiment the control cells were placed in fresh

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[3] Abbreviations: APW: artificial pond water, number following APW indicates pH of solution (20); CCCP: carbonyl cyanide *m*-chlorophenyl hydrazone; DCCD: dicyclohexylcarbodiimide; DES: diethylstilbestrol; DMO: 5,5-dimethyloxazolidine-2,4-dione; DNP: 2,4-dinitrophenol; EDAC: ethyl-3-(3-dimethylaminopropyl)carbodiimide.

solution and the others transferred to a solution containing the inhibitor. The appropriate time of exposure to the inhibitor was determined from previous electrical measurements (5) as the time needed to give a steady membrane potential and resistance.

Following incubation, the cells were individually dipped into liquid N_2 until frozen. They were immediately crushed in 2.0 ml of ice-cold 5.0% trichloroacetic acid containing 3.0 mM EDTA. This extract was well mixed, the cell debris centrifuged down, and the supernatant stored on ice until assayed.

The assay was done by adding to the 4.0 ml of assay buffer in the scintillation vials, either 50-μl of cell extract or 100 μl of ATP standard. The luciferin-luciferase enzyme was added as a drop of 50 μl to the side of the vial, and the reaction timing started when this was mixed into the assay buffer. The vial was counted in the scintillation counter for 30 s beginning 30 s after the start of the reaction. This 30-s delay allowed time to put the sample in the counter, and for the rapid phase of decay of counts to conclude (which was mostly due to exposure of the vial and solution to room light). The counting period was in the long slow exponential phase of the decay of counts. The calibration curve was linear and reproducible over a range greater than that found in the samples.

The Beckman scintillation counter was set at a gain between 0.11 and 0.20, as determined by the level of activity of the enzyme for the day, and a combined ^3H and ^{14}C window was used. The enzyme activity declined by about 10% over the 3 h needed to analyze a set of samples, so standards were run between determinations for each sample. Since the trichloroacetic acid used in extracting the cells quenches the counts, an equal amount was added to the ATP standard. Tissue extract also caused quenching but this was less than 4% and was smaller than the variability between repeats of the same sample, and was consequently ignored.

The results were calculated as concentration of ATP in the cytoplasm, knowing that the ATP was in the cytoplasm (A. G. Miller, personal communication) and assuming that the cytoplasm is 5% of the cell volume (12).

Electrical measurements were made as described previously (5).

RESULTS AND DISCUSSION

The ATP concentrations for cells in the different inhibitors are presented in Table I, and are also given as a percentage of the simultaneously measured controls. The large variation of the ATP concentration of the controls was disturbing but cannot be attributed to any of the assay procedures. This variation probably represents actual changes in ATP concentrations in the culture over a period of several months.

The changes in ATP concentrations caused by these inhibitors will be compared with measurements of membrane potential, membrane resistance, and potassium permeability that were reported in an earlier paper (5).

Uncoupling Agents. In the light, 5.0 μM CCCP caused the membrane potential to depolarize to a diffusion potential and the membrane resistance to increase. Before the initiation of these changes there is a lag time of about 3 ks. In the dark, the final results are the same but the membrane potential begins to depolarize immediately after the addition of CCCP and the membrane resistance decreases slightly before the increase begins (5).

The change in membrane potential makes an interesting parallel to the change in ATP concentration. In the light both the ATP level and membrane potential decay more slowly than in the dark (see Fig. 1). While the time courses of the fall of ATP level and membrane potential depolarization differ between the light and the dark, the final steady value attained by each parameter is the same in both the light and the dark. Greater reductions of the ATP level in the dark than in the light under CCCP inhibition have been observed previously in *Griffithsia* (8) and *Chara foetida* (13).

These effects of CCCP on the ATP level suggest two possible interpretations: (a) the mitochondria are more sensitive to the effect of CCCP than are the chloroplasts; and (b) in the light, with a large capacity for synthesis of ATP, the cell can withstand the effects of CCCP for a longer time before the ATP level falls. The first possibility is substantiated by Heytler and Pritchard (2) who found that extracted chloroplasts are 50% inhibited by 1.3 μM CCCP while extracted mitochondria are 50% inhibited by only 0.3 μM CCCP. Since the two above possibilities are not mutually exclusive, both could combine to give the observed effect.

The final ATP concentration in both the light and the dark with CCCP is 10% of the control (Table I) and appears to be a low enough value to inhibit the electrogenic ion pump.

At a concentration of 0.1 mM, DNP in the light has a similar but more rapid effect than CCCP on the membrane potential and resistance (5). DNP reduced the ATP concentration to 32% of the original value (Table I). It is possible that higher concentrations of DNP would have lowered the ATP level still further to a value similar to that produced by CCCP. However, further inhibition of the electrogenic pump would not be expected to depolarize the

Table I. *ATP concentration as affected by different inhibitors with membrane electrical properties for comparison*

The column labeled "time" indicates how long the cells had been in the inhibitor. The column labeled "control" gives the ATP concentration in uninhibited cells measured in the same experiment. The values in the last three columns are from a previous paper (5). The experiments are numbered in the order performed. Experiments 6 and 7 were done with cells from a different culture tank than used for the other experiments. Results are expressed as mean ± standard error of the mean (number of cells measured).

Experiments	Inhibitor	Time	Control	Treatment.	[ATP] % of Control	Membrane Resistance	Membrane Potential	Diffusion Potential
		ks	*mM ATP*	*mM ATP*		*kohm/cm²*	*mv*	*mv*
4)	5.0 μM CCCP, light	2.0	3.06 ± 0.35 (9)	1.65 ± 0.25 (9)	54%	9 ± 1 (10)	−194 ± 4 (10)	−103
		7.5	3.06 ± 0.35 (9)	0.30 ± 0.06 (10)	10%	42 ± 8 (9)	−93 ± 8 (9)	−82
5)	5.0 μM CCCP, dark	2.0	1.36 ± 0.17 (9)	0.17 ± 0.02 (9)	12%	23 ± 5 (8)	−143 ± 7 (8)	−122
		7.5	1.36 ± 0.17 (9)	0.13 ± 0.02 (10)	10%	52 ± 9 (8)	−93 ± 7 (8)	−105
2)	0.1 mM DNP, light	3.0	1.92 ± 0.23 (9)	0.61 ± 0.04 (9)	32%	50 ± 5 (15)	−115 ± 4 (15)	−109
1)	50 μM DCCD, light	5.0	2.53 ± 0.41 (8)	0.72 ± 0.15 (8)	29%	75 ± 7 (13)	−144 ± 3 (13)	−123
1)	40 μM DES, light	4.0	2.53 ± 0.41 (8)	0.38 ± 0.12 (9)	14%	122 ± 13 (9)	−99 ± 3 (9)	−106
3)	1.0 mM EDAC, light	3.0	1.69 ± 0.19 (9)	1.69 ± 0.18 (10)	100%	10 ± 1 (5)	−142 ± 5 (5)	−109
2, 8)	2.0 μM DCMU, light	4.5	2.71 ± 0.18 (19)	3.05 ± 0.23 (20)	113%	43 ± 7 (8)	−186 ± 9 (8)	−107
9)	Darkness	5.4	2.01 ± 0.07 (9)	2.15 ± 0.10 (10)	107%	43 ± 9 (12)	−193 ± 4 (15)	−106
6)	5.0 mM DMO, light	2.0	3.99 ± 0.28 (10)	3.59 ± 0.28 (10)	90%	12 ± 1 (12)	−194 ± 3 (12)	
		7.5	3.99 ± 0.28 (10)	3.51 ± 0.25 (10)	89%	15 ± 3 (4)	−160 ± 6 (15)	−100
7)	5.0 mM NH_4Cl, light	1.0	1.81 ± 0.15 (10)	1.54 ± 0.14 (9)	85%			
3)	0.5 mM $LaCl_3$, light	9.0	1.69 ± 0.19 (9)	1.76 ± 0.19 (10)	104%	12 ± 2 (8)	−129 ± 8 (9)	−86
	Control					15 ± 1 (37)	−189 ± 3 (38)	−106

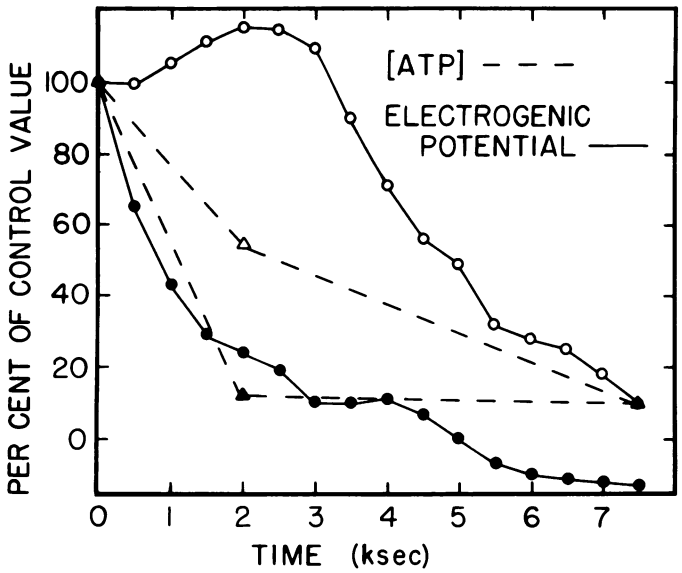

FIG. 1. Decrease in ATP level in *C. corallina* during exposure to 5.0 μM CCCP in the light (△) and in the dark (▲) as compared with the electrogenic potential in the light (○) and in the dark (●). Electrogenic potential is calculated from observed potential by subtracting the expected diffusion potential and expressing this as a per cent of the initial value (data from ref. 5).

membrane potential below the passive diffusion potential (0.1 mM DNP lowered the membrane potential to −110 to −112 mv compared to the calculated diffusion potential of −109 mv [5]).

ATPase Inhibitors. DCCD is a rather unspecific inhibitor of membrane-bound ATPases (15). Thus, it might be expected to inhibit not only the postulated ATPase-electrogenic pump in the plasmalemma but also the ATPases in the mitochondria and chloroplast membranes. Once these ATPases are inhibited, other ATPases not affected by DCCD will quickly use up the ATP, lowering the concentration in the cell. The observed effect of 50 μM DCCD is a lowering of the ATP level to 29% of the control (Table I). The same concentration caused inhibition of the electrogenic ion pump (5).

DES, another inhibitor of membrane-bound ATPases (1), caused the ATP level to drop to 14% of control (Table I). At the concentrations used, DES is more effective and faster acting than DCCD. DES caused depolarization of the membrane potential and an increase in membrane resistance, indicating that the electrogenic ion pump had been inhibited (5).

The compound EDAC is a carbodiimide, as is DCCD, and is likewise expected to attack ATPases (9). While EDAC did depolarize the membrane potential, it caused the membrane resistance to decrease instead of the increase expected if the pump had been inhibited (5). These results are not consistent with the inhibition of an electrogenic pump with conductance. The absence of an effect of 1.0 mM EDAC on the ATP level (Table I) suggests that this water-soluble compound may not cross the membrane and thus could not inhibit ATP production and use. This would limit the action of EDAC to the cell surface, where it may affect Ca^{2+} binding (14). A reduction of Ca^{2+} binding could cause the observed increase in potassium permeability and consequently the membrane depolarization and decrease in membrane resistance (5).

DCMU and Darkness. If, as Smith and Raven (19) suggested, *C. corallina* does not have functional cyclic photophosphorylation, or energy from this source is not available for use by transport processes, then DCMU, an inhibitor of noncyclic photophosphorylation, should have the same effect as darkness. Electrically, both darkness and DCMU have the same effect, the potential remains basically unchanged, and the resistance increases (5). Although both DCMU and darkness should inhibit production of ATP, in neither case was there a significant change in the ATP level ($P < 5\%$; Table I). The effect of DCMU on the electrogenic pump and ATP levels seems to mimic the effects of darkness. The increase in membrane resistance with both darkness and DCMU indicates an alteration of the properties of the electrogenic pump that occurs without any apparent change in the ATP levels. This suggests a control mechanism affecting the pump that operates independently of the ATP levels.

DMO and NH_4Cl. DMO is a weak acid that is permeable in its neutral form and in high concentrations its dissociation inside the cell is sufficient to lower the internal pH (21). The effect of DMO on ATP production should be small. The observed change was a decrease that is not significant compared to the uncertainty of the measurements (Table I). Thus, in *Chara*, DMO has not only a minimal effect on membrane potential and resistance (5) but also little effect on the ATP level.

The ion NH_4^+ has been used as an uncoupler of photophosphorylation (6). It probably penetrates the membrane most rapidly in its neutral form and, being a weak base, may raise the internal pH as it associates with H^+. If the electrogenic ion pump is an H^+ efflux pump that regulates internal pH, NH_4Cl should stop the operation of the pump by raising the internal pH of the cell, since the pH cannot be restored by the pump and its H^+ efflux. This should cause a depolarization of the membrane potential. This is observed; a substantial depolarization occurred 100 to 200 s after the addition of NH_4Cl. The only complication is that since NH_4^+ is an uncoupler of photophosphorylation, this depolarization could be due to a decrease in energy supply to the pump. However, even a 1.0-ks exposure to 5.0 mM NH_4Cl caused only a 15% decrease in ATP levels. This is an ATP level only slightly lower than that due to an exposure of 2.0 ks to DMO, and this exposure did not cause a significant depolarization. Kurkdjian *et al.* (7), using the DMO method, have shown that the cytoplasmic pH of *Acer pseudoplatanus* cells was elevated by up to 0.3 pH units for as long as 3 h after exposure to 20 mM NH_4Cl but then became more acid. If the same effect occurs in *C. corallina*, the slight decrease in cytoplasmic pH observed by Walker and Smith (24) after a 15-h exposure of the cells to 2 mM NH_4Cl may not be relevant for the short exposures used here. Thus, the rapid effect of NH_4Cl on the potential may be ascribed to an elevation of cytoplasmic pH rather than an effect on the ATP level.

Lanthanum. Even a long (9.0-ks) incubation in 0.5 mM $LaCl_3$ caused no significant change in the ATP levels. Lanthanum, which drastically lowers K^+ permeability of the membrane, causes the membrane potential to depolarize, but has little effect on the membrane resistance (5). Due to its large charge, lanthanum should be less permeable than Ca^{2+} which enters cells only slowly (22). Thus, it is unlikely that significant amounts of lanthanum enter the cell and it is not surprising that it has no effect on internal ATP levels.

Relationship between ATP Levels and Electrogenic Pump. If the electrogenic ion pump in *C. corallina* is an ATPase as postulated, lowering the ATP level in the cell should inhibit the pump. In this paper, the following inhibitors were found to lower the ATP level significantly: CCCP, DNP, DCCD, and DES. In all cases, these compounds in the same length of time and at the same concentration have also been shown to depolarize the membrane potential to a diffusion potential and increase the membrane resistance from 15 kohm/cm^2 to values of 60 kohm/cm^2 and above. The changes in these two parameters indicate that the pump has been inhibited (5, 20).

The other inhibitors, EDAC, DCMU, darkness, DMO, NH_4Cl, and $LaCl_3$, did not drastically change the ATP concentration. None of these compounds had a large effect on the membrane resistance, never increasing the resistance above 45 kohm/cm^2, and usually the resistance was much lower. This indicates that the pump is still operating. DCMU, darkness, and DMO did not have

a large effect on the membrane potential, again indicating that the pump is operating. $LaCl_3$ caused a depolarization of the membrane potential, but still the membrane potential is more hyperpolarized than the diffusion potential (5). EDAC depolarized the membrane but since there was no concurrent increase in the membrane resistance and no change in ATP levels, this effect has been postulated as due not to electrogenic pump inhibition but to an over-all increase in membrane permeability, as evidenced by an increase in K^+ permeability (5), perhaps caused by the effect of EDAC on Ca^{2+} binding. This would short circuit the pump and depolarize the membrane potential to a value between the normal potential and the diffusion potential. Finally, the large depolarization caused by NH_4Cl, to a value near the diffusion potential, was explained in terms of its effect on the pH regulating ability of the pump since it does not appear to act as an uncoupler under these conditions.

The obvious conclusion from this work is that the electrogenic pump is an ATPase. However, ATP could be acting as an activator of the pump, or may be needed to maintain the integrity of the membrane and proteins for transport activity, instead of or in addition to being a direct energy source for the electrogenic pump. The results reported here cannot distinguish between these possibilities.

Evidence for ATP being the energy supply for an H^+ electrogenic pump comes from the work of Walker and Smith (24). They show that the energy available from ATP closely matches the energy in the electrochemical gradient of H^+ over the range of pH from 5 to 7. Shimmen and Tazawa (17), by internal perfusion of internodal cells of *Chara australis* with various levels of ATP and Mg^{2+}, find changes in membrane potential and resistance which provide evidence for an electrogenic Mg^{2+}-activated ATPase in the membrane.

Our results are similar to the findings in *Neurospora* (18) where there is a good correlation between a decrease in the ATP concentration and the depolarization of the membrane. Both in *Neurospora* and in *Chara* this provides evidence for an electrogenic ATPase in the plasmalemma. It is likely that each of these pumps transports H^+ but two major differences have been observed between the plant and fungal electrogenic pumps. First, in *Neurospora* the conductance through the pump is small compared to the total conductance through the membrane. In *Chara* the pump conductance is more than an order of magnitude larger than the other conductances through the membrane. Second, in *Neurospora* (18) the coupling ratio appears to be 1 H^+/ATP, while in *Chara* it is probably 2 H^+/ATP (24). These two observations are probably interrelated. In *Chara* the energy available from ATP is equal to the H^+ electrochemical gradient so the electrogenic pump is stalled under normal conditions. When current is passed through the membrane the change in the membrane potential alters the flux through the electrogenic pump and contributes to the measured membrane conductance. In *Neurospora*, with a coupling ratio of 1 H^+/ATP, the pump is not stalled and its rate of operation is only slightly altered by changes in the membrane potential, thus when the membrane conductance is measured the pump makes only a small contribution to the total conductance. The implication is that the rate of the electrogenic pump in *Neurospora* must be controlled by some factor other than the electrochemical potential gradient.

In conclusion, we feel that the hyperpolarization of the membrane potential beyond the diffusion potential in *C. corallina* is due to an electrogenic ion pump. This pump appears to be an ATPase located in the plasmalemma that contributes significantly to the membrane potential and is the major conductance in the membrane. Inhibition of the pump causes both a depolarization of the membrane potential and a decrease in the conductance of the membrane. Inasmuch as it is likely that this pump carries H^+, more work needs to be done to determine how this pump is controlled by or regulates the internal pH.

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