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Evidence for Electrogenic Proton Extrusion by Subepidermal Cells of *Lemna paucicostata* 6746

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Abstract. The cell potential of *Lemna paucicostata* 6746 was measured between the vacuole and the external solution. The potential in the dark (−202 mV) could be depolarized with 0.1 mM dicyclohexyl carbodiimide (DCCD) or 1 mM arsenate to −81 mV. The hyperpolarization above the latter value is therefore attributed to an ATP-dependent process. The cell potential showed a significant dependence upon the pH of the external solution. The change in the potential induced by a jump in pH between two certain values, was reversible and independent of the mode of performing the pH change (stepwise or at once). The DCCD- or arsenate-depolarized potential did not respond to external pH changes. A 0.1 mM ammonium chloride solution depolarized the cell potential reversibly to −83 mV. This potential-change could be greatly reduced by simultaneous addition of 5 mM Na isobutyrate. The pH sensitivity of the cell potential is ascribed to changes in the rate of proton extrusion upon altering the proton gradient across the plasmalemma. The effects of ammonium and isobutyrate are interpreted as being the consequence of pH shifts at the inner face of the plasmalemma, caused by the permeation of the undissociated form of the weak acid or base. A critical discussion of an alternative interpretation for the ammonium effect is presented.

Key words: *Lemna* – Membrane potential – Proton extrusion.

Introduction

The vacuolar potential of subepidermal cells of *Lemna* is about −200 mV (Löppert et al., 1978; Novacky et al., 1978). As in various other plants, this potential is considerably higher than would be expected from passive diffusion of ions (e.g., Findlay and Hope, 1976). It is now well established that the active component of the membrane potential (i.e., that fraction which is sensitive to inhibitors of energy metabolism) is generated by electrogenic transport of ions (Bentrup et al., 1973; Spanswick, 1973; Anderson et al., 1974, 1977; Higinbotham and Anderson, 1974; Poole, 1974; Gradmann, 1976). Protons have been considered to play a dominant role in this process (Raven and Smith, 1976, 1977). A direct proof that H^+ is the ionic species involved in membrane hyperpolarization should be possible from a correlation of the membrane potential and the proton flux rate. However, experimental verification encounters appreciable difficulties: Proton fluxes evade direct measurement, and they cannot be induced or stalled simply by addition or removal of H^+ at the plasmic side of the plasmalemma. The intent of the present investigation was to manipulate proton pumping by changing the pH at both sides of the plasmalemma and to monitor the accompanying changes of the membrane potential.

Abbreviation: DCCD = N,N′-dicyclohexyl carbodiimide

Materials and Methods

Lemna paucicostata Hegelm., strain 6746, was cultured under aseptic conditions as described previously (Löppert et al., 1978); the daily light phase was 16 h (long-day conditions).

Membrane potential measurements were carried out according to the usual electrophysiologic methods. The micro plant chamber, described previously (Löppert et al., 1978), was modified in one respect: The bathing solution was conducted via a capillary tube to the lower surface of the plant. Because the volume of the chamber was small (about 20 µl), the bathing solution could be changed rapidly.

The bathing solution, designated as solution X in Figure 2, contained 4 mM KNO_3, 1.5 mM KH_2PO_4, 5.5 mM $CaCl_2$, and

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1.2 mM $MgSO_4$, unless otherwise stated. Its pH was set to 5.0 and was continuously checked with a micro pH-electrode in the outlet of the plant chamber.

Ammonium uptake was calculated from the rate of its disappearence in the medium. Aliquots of 1 ml were removed and after addition of 10 µl 10 M NaOH, the samples were immediately assayed for NH_3 with a gas-sensitive electrode.

Results

The cell potential showed reversible changes when the pH of the bathing solution was changed (Fig. 1). Whether the pH-jumps between pH 7 and pH 4 were carried out stepwise or at once, had no influence upon the stationary-state potentials. When the cell potential was depolarized in the dark with 0.1 mM DCCD or 1 mM arsenate, it was no longer sensitive to pH changes in the medium. (The arsenate effect is not included in Fig. 1.)

Addition of NH_4Cl to the medium caused an instantaneous depolarization in the light as well as in the dark (Fig. 2). No effect was observed when the cell potential was depolarized previously to the addition of ammonium in the dark and by the presence of 0.1 mM DCCD or 1 mM arsenate (not included in Fig. 2). The depolarizing effect of ammonium could be reduced drastically by simultaneous addition of Na isobutyrate. In contrast, Na isobutyrate itself caused only a small hyperpolarization. The cell potential did not respond to addition of NaCl (5 mM final concentration).

The rate of ammonium uptake into *Lemna* was 4.7 µmol g^{-1}FW h^{-1} in the light at pH 5 and an external NH_4Cl concentration of 0.1 mM.

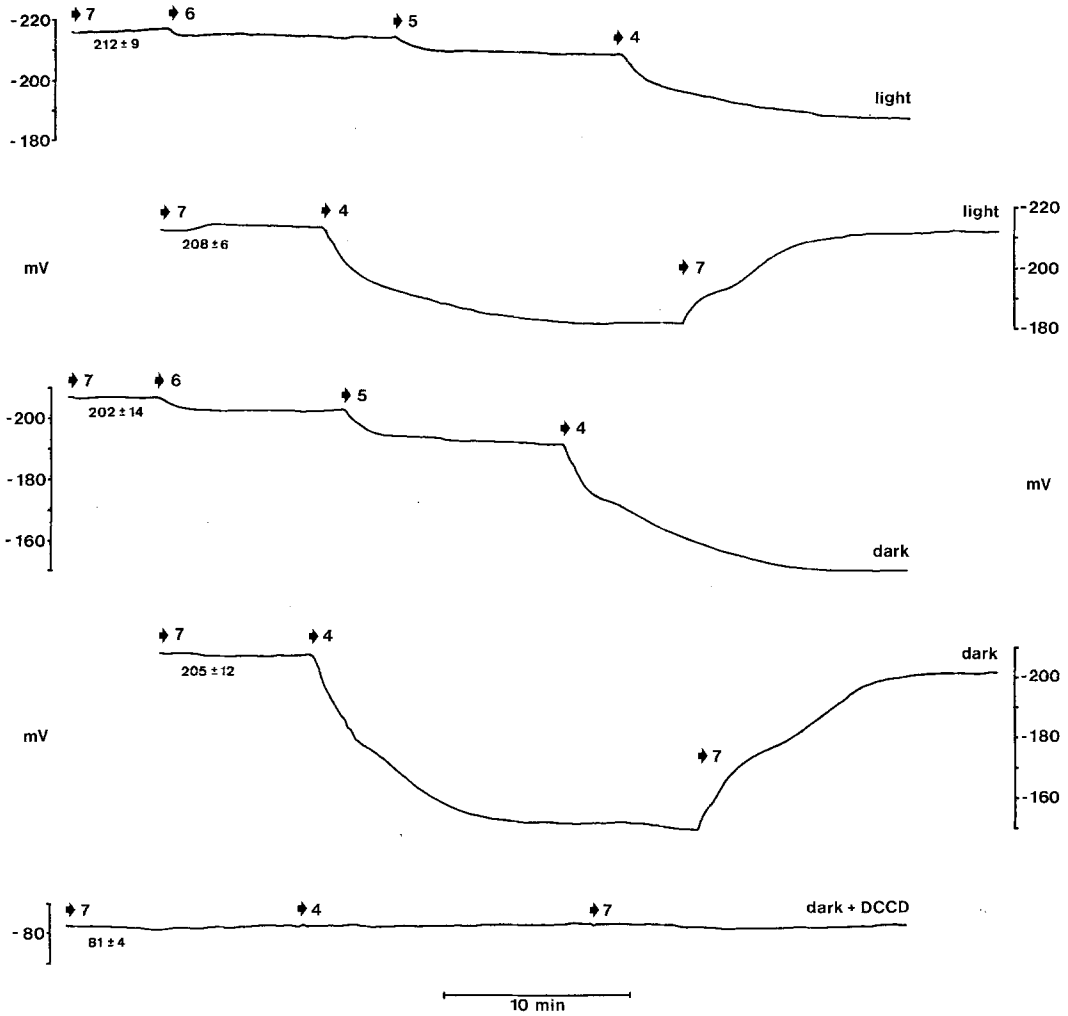

Fig. 1. The effect of external pH changes on the cell potential. The pH changes in the bathing solution were achieved by rapid switch-over between solutions of different pHs, as indicated by the *arrows* and *figures* above the recorder trace. In these experiments, KH_2PO_4 has been omitted from the bathing solution. The figures below a certain part of the recorder trace are mean values (± = standard deviation), calculated from corresponding potentials of at least four different experiments

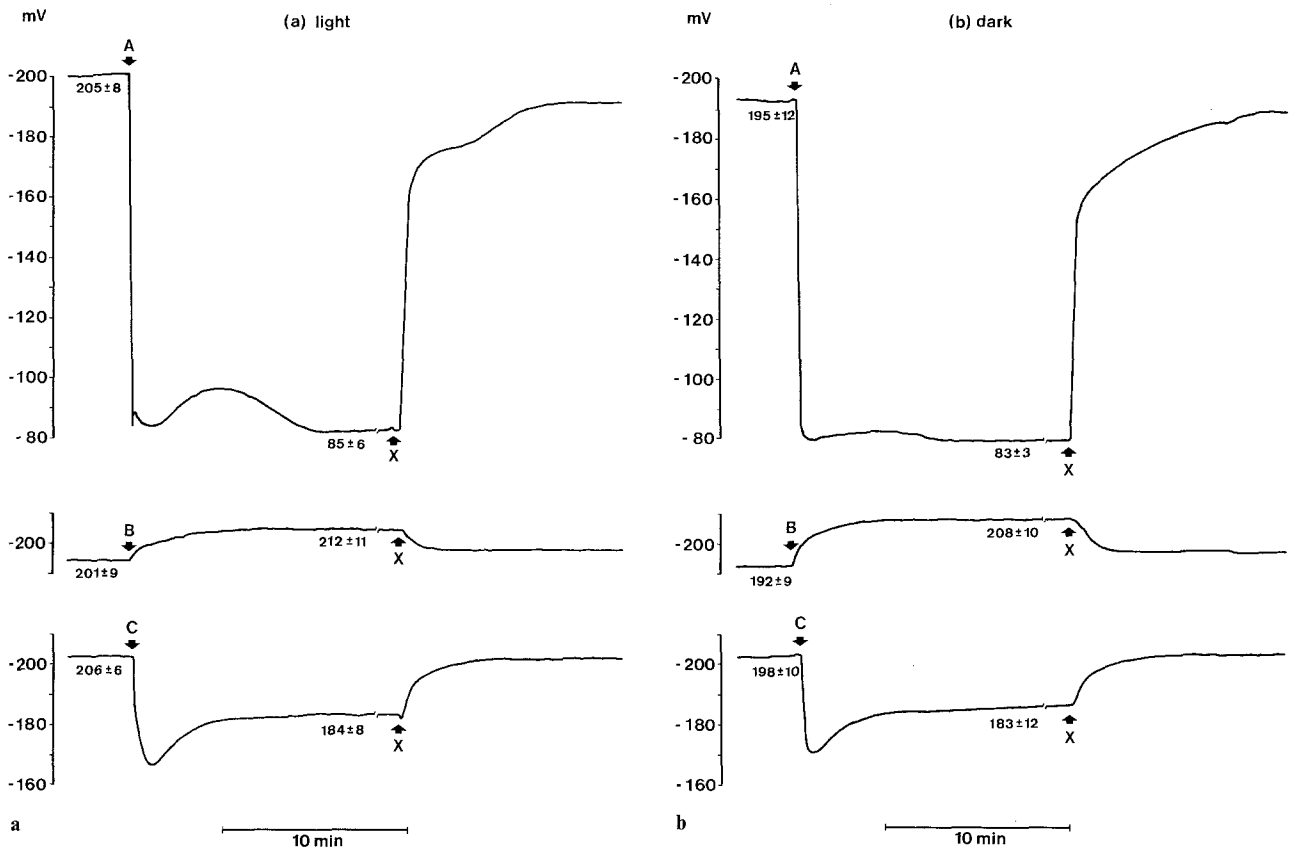

Fig. 2a and b. The effect of ammonium and isobutyrate on the cell potential. All experiments were started with the plants being flushed with solution X (4 mM KNO_3, 1.5 mM KH_2PO_4, 5.5 mM $CaCl_2$, 1.2 mM $MgSO_4$). At the time indicated, the solution was changed to one which additionally contained: 0.1 mM NH_4Cl (*A*), 5 mM Na isobutyrate (*B*), 0.1 mM NH_4Cl+5 mM Na isobutyrate (*C*). The figures below a certain part of the recorder trace are mean values (± = standard deviation), calculated from corresponding potentials of at least four different experiments

Discussion

An important point in the discussion of mechanisms that lead to the generation of membrane potentials is the extent to which active and passive processes are involved. Blocking of energy transduction with various inhibitors is commonly employed for this purpose. Two substances that interfere with ATP generation were used in this investigation. DCCD is known to inhibit membrane-bound ATPase (Harold et al., 1969), and arsenate interferes with phosphorylation reactions (Crane and Lipmann, 1953; Weigl, 1963). Because both inhibitors depolarized the cell potential in the dark to a low value (Fig. 1), hyperpolarization above the latter in the absence of the inhibitors is probably due to ATP-dependent processes. It should suffice at present to state that this conclusion is limited to experimental conditions in which the plants are kept in darkness; illumination reverses the inhibitor-mediated depolarization to a great extent (Löppert, unpublished).

From the fact that the cell potential showed no response to changes in external H^+ concentration when energy transduction is blocked (Fig. 1: dark+DCCD), it follows that the passive permeability of protons is small in comparison to that of other ions, so that diffusion of H^+ does not contribute measurably to the diffusion potential. Therefore, the changes of cell potential accompanying the pH changes of the bathing medium could be attributed to changes in the rate of active processes. It seems most plausible to regard proton pumping itself as the active process under consideration. The dependence of the cell potential on external pH is then consistent with a model discussed by Spanswick (1973, 1974). He ascribes a considerably higher conductivity to the proton pump than to the passive channels in parallel; consequently, the effect of external pH on the cell potential is an effect on the electromotive force of the pump rather than on the diffusion potential.

The extrusion of H^+ from cells has been attributed

a predominant role in the regulation of cytoplasmic pH (Raven and Smith, 1976). Therefore the pump rate should respond in a very sensitive way to changes in cytoplasmic pH. To achieve such changes, membrane-permeant bases and acids have been used; this method has been recently employed to induce intracellular pH changes in *Chlorella* (Tromballa, 1978). The undissociated form of these compounds is known to penetrate biologic membranes by diffusion (Collander, 1959). Upon reequilibration with their ionic forms on either side of the membrane, protons are consumed or released. This leads to pH changes, probably occurring most rapidly and most pronounced in the ultimate vicinity of the membrane. Because this is the region "viewed" by the postulated membrane-bound proton pump, H^+ extrusion should be affected by these substances.

The effect of ammonium on the cell potential (Fig. 2) seems to justify the above considerations in a distinct way. Upon diffusion through the plasmalemma as NH_3 and subsequent reequilibration with NH_4^+, protons are consumed at the inner face of the membrane, thus leading to an increase in pH. As a consequence, proton efflux ceases and the membrane potential is diminished by the amount due to proton pumping.

However, precaution is necessary when interpreting the depolarizing effect of NH_4Cl. An alternative explanation could be based on a mechanism proposed for the uptake of methyl ammonium and ammonium by *Hydrodictyon* and *Chara* cells (Smith and Walker, 1978; Smith et al., 1978). Methyl ammonium and ammonium are thought to enter the cell in their protonated forms by uniport. Thus, the inward-directed current depolarizes the cell potential. In the case of *Lemna* a similar mechanism should not be excluded; however, ammonium uniport could be ruled out as the cause for the dramatic effect of NH_4Cl on the membrane potential for the following reasons.

Firstly, the depolarization of the cell potential after addition of NH_4Cl could be greatly reduced when Na isobutyrate was added together with ammonium (Fig. 2). This is probably due to the function of isobutyric acid as a protonophor, diminishing the alkalinization caused by NH_3 permeation. Secondly, the observed magnitude and kinetics of cell depolarization after NH_4Cl addition are quite unexpected in the case in which a NH_4^+-uniport or NH_3-H^+-symport is assumed. This becomes evident from a comparison of the effect of ammonium with the change in potential, induced by other species known to be transported electrogenically. For example, hexose is taken up into *Lemna* by a H^+-hexose cotransport system (Novacky et al., 1978). The influx rate of glucose is as much as 7.5 μmol g^{-1}FW h^{-1}, which is similar to the uptake rate of ammonium (see Results). The effects of glucose and ammonium on the cell potential are quite different: A transient depolarization of about 40 mV after glucose addition (Novacky et al., 1978) is contrasted with a lasting depolarization of more than 100 mV in the case of ammonium (Fig. 2). (A slowly increasing potential was observed only after 20 min.) Thus it seems that the ammonium-effect observed with *Lemna* is due to a decrease in proton efflux, rather than to a depolarizing current. It should be noted that the situation with *Lemna* is obviously different from that encountered with *Chara* or *Hydrodictyon*. A rough estimate of the flux of ammonium into *Lemna* gives a value of $J_{NH_3(+NH_4^+)} = 4.3$ nmol $m^{-2} s^{-1}$ (a mean cell diameter of 20 μm has been assumed). This is about 6% of the methyl ammonium influx into *Chara* (72.8 nmol $m^{-2} s^{-1}$ in the light at pH 5.5, according to Smith and Walker, 1978).

In conclusion, the assumption that the active component of the cell potential of *Lemna* is caused by electrogenic proton extrusion, is consistent with the available evidence. Further investigation is being carried out on mechanisms regulating the proton pump.

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