Circulation of Potassium Across the Plasma Membrane of *Blastocladiella emersonii*: K⁺ Channel

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A previous paper reported that the water mold *Blastocladiella emersonii* generates a transcellular electrical current, such that positive charges enter the rhizoid and leave from the thallus (Stump et al., Proc. Natl. Acad. Sci. U.S.A. 77: 6673-6677, 1980). To begin to understand the genesis of this current we investigated ionic relationships in this organism by use of intracellular microelectrodes. In cells suspended in buffered CaCl₂, the membrane potential could be accounted for as a K⁺ diffusion potential; no evidence for an electrogenic pump was obtained. Potassium ions diffuse outward by a pathway that also carries Rb⁺ and Ba²⁺, but excludes both smaller and larger ions (Li⁺, Na⁺, Cs⁺, Mg²⁺, Ca²⁺, and choline). Chloride and other anions make little contribution to the potential, but the presence of Ca²⁺ in the external medium is required for successful potential measurements. In growing cells, the internal K⁺ concentration is generally somewhat higher than would be expected if the K⁺ distribution were determined entirely by the membrane potential. Under certain conditions, net uptake of K⁺ against the electrochemical potential gradient was observed. We suggest that K⁺ is actively accumulated by a primary transport system that may exchange K⁺ for H⁺, and that K⁺ leaks passively outward through the K⁺ channel. The K⁺ circulation across the membrane amounts to about 2% of the K⁺ pool per min, or 4.5 $\mu\text{A}/\text{cm}^2$ of surface area. We propose that this K⁺ circulation is one arm of the transcellular current, carrying positive charge out of the thallus.

The water mold *Blastocladiella emersonii* is known to biologists chiefly because of its spectacular developmental cycle (8). However, it also deserves attention as a primitive and quite tractable single-celled eucaryotic organism that is structurally and functionally polarized. During the growing phase of the life cycle, the organism (Fig. 1) consists of two quite distinct regions. One is the bulbous thallus, 50 to 75 μm in length, which contains all the nuclei and the bulk of the cytoplasm and is destined eventually to turn into a sporangium. The other is the rhizoid system, a mass of filaments that usually sprout from the bottom of the thallus. The rhizoids are sometimes described as a holdfast and do indeed help attach the organism to solid surfaces, but their primary physiological function is probably to transport nutrients to the thallus. Previous research from this laboratory (4) showed that rhizoidal filaments grow chemotropically towards sources of phosphate and of amino acids. In liquid culture two thirds of the cell's phosphate is taken up via the rhizoids, and conditions can be so arranged that all the phosphate enters by this route; by contrast, potassium and calcium ions enter thallus and rhizoids equally (D. L.

Kropf, unpublished data). That the rhizoids serve the same function in nature is suggested by the habit of *Blastocladiella* of growing attached to bits of vegetable debris.

The point of departure for the present study is the discovery (22) that *B. emersonii* is electrically polarized. Growing organisms generate an endogenous transcellular electrical current, of the order of 1 $\mu\text{A}/\text{cm}^2$, such that positive charges enter the rhizoids and leave from the thallus; during sporulation the current reverses direction, with positive charges entering the thallus and leaving from its basal region. The physiological role of the transcellular current is not known; we suspect that it is involved in the orientation of rhizoid growth (4) and perhaps in the translocation of nutrients.

This communication is concerned with the ionic mechanisms that generate the transcellular current. By analogy with *Neurospora crassa* and with yeasts, we expected to find that protons are expelled from the thallus by an electrogenic proton pump, and hoped to account for the outward current on this basis (17, 18, 20; see also reviews in references 3, 3a, 14, and 19). In fact, the present studies with intracellular micro-

FIG. 1. Vegetative cell of *B. emersonii* with microelectrode in recording position. Bar, 50 μ m.

electrodes produced no evidence for an electrogenic proton pump. Instead, the transmembrane electrical potential apparently arises by the diffusion of potassium ions out of the cell through a specific pathway or channel. The electrochemical K^+ gradient that underlies the diffusion potential is probably established by a primary active transport system that allows the cell to accumulate K^+ , perhaps by exchange for protons.

MATERIALS AND METHODS

Organism and growth media. *B. emersonii* L-17 was a gift from David Sonneborn and was maintained in the form of resistant sporangia as described by Lovett (7). Vegetative cells were grown on one of the following media. PYG is a complex medium (1.25 g of peptone [Difco], 1.25 g of yeast extract [Difco], and 3.0 g of glucose per liter, adjusted to pH 7 with NaOH; when prepared in this manner, the K^+ concentration is 1.1 mM). DM2 is a defined medium (16, 21), containing 25 mM K^+ , 13 mM Na^+ , 1 mM Ca^{2+} , and 10 mM Mg^{2+} , plus phosphate, trace metals, amino acids, and thiamine. DMP is a modified version of DM2 (22) containing 0.5 mM each of K^+ , Ca^{2+} and Mg^{2+} but no Na^+ ; it is not an optimal medium for growth but is useful when a low ionic strength is desired. Zoospores were produced by standard methods, slightly modified (21, 22, 26).

For electrophysiology, the cells were grown attached to the bottom of plastic dishes (Falcon tissue culture dish, 60 by 15 mm); each dish contained 4 ml of medium and was inoculated with 5,000 zoospores (21, 22). The dishes were incubated at 24°C, and the cells

were examined after 16 to 20 h. Membrane potentials were measured either in the original growth medium or after replacement of the medium by repeated rinsing with sporulation solution (SS; 21). This consists of 1 mM Tris-maleate and 1 mM $CaCl_2$ (pH 6.8). Modifications of this buffer for particular experiments are given below; free calcium concentration was calculated using the equations and stability constants described by Owen (13).

Experiments on potassium transport and intracellular pH were done with cells grown in liquid suspension, essentially as described by Lovett (7, 8). The medium (500 ml in a jacketed spinner flask) was inoculated with 5×10^4 zoospores per ml, plus 2 drops of Antifoam B (Sigma), and aerated at 24°C overnight. Strict attention to aseptic conditions was mandatory. Under these conditions, overnight growth in PYG medium yields 0.5 to 1.5 mg (dry weight) of cells per ml. Clumping of the cells was generally not excessive, but could not be prevented altogether. The cells were either used directly or else collected by filtration through Sargent 500 paper, washed, and resuspended in SS or another medium. To break up clumps before use, the suspension was gently shaken on a rotary shaker for 15 min.

Membrane potential. *B. emersonii* forms large multinucleate cells, 50 μ m or more in diameter, that are easily impaled with microelectrodes (Fig. 1). The microelectrodes were made with Kwik-fil capillary tubing (1.2 or 1.5 mm; WPI Instruments, New Haven, Conn.) and were filled with 3 M KCl or potassium acetate; electrode resistance ranged from 5 to 20 Mohms. A second microelectrode, or else a commercial one from Microelectrodes, Inc. (Londonderry, N.H.), served as reference electrode; tip potentials did not exceed 5 mV, and were ignored. Impalement was routinely done with the aid of an "aus Jena" micromanipulator (Jena, German Democratic Republic). In early experiments, potentials were measured with a Radiometer PHM26 pH-meter in the DSEP mode and recorded on a chart recorder. Subsequently we used a KS-700 Dual Micro-Probe electrometer (WPI Instruments).

When a cell is impaled, the potential normally drops to its steady-state level within 10 s and remains constant thereafter. Sometimes, however, we saw periodic "pulsing" of the potential: it rose almost to zero and, after a few seconds, returned to the previous value. Some cells pulsed constantly for minutes on end, others not at all. Since action potentials are known to occur in *N. crassa* and various algae, we thought that the pulsing might be a biological phenomenon. However, we are now persuaded that it is an artifact akin to those noted by Slayman (17; personal communication). Pulsing was often seen in cells impaled with electrodes drawn from 1.5-mm tubing, very rarely when 1.2-mm tubing was used; even in the former case, pulsing was eliminated by filling the electrodes with 0.75 M potassium citrate in place of KCl. The value of the steady membrane potential was unaffected by the size of the tubing used; nevertheless, we now routinely use the 1.2-mm tubing.

It is possible to impale a cell and then to exchange the medium for another by use of a pair of coupled syringes. However, since the thallus is not firmly attached to the substratum, most such experiments ended with loss of the electrode from the cell. For this

reason, most of the data reported here were gathered from a number of individual cells, and then pooled.

Membrane resistance. Membrane resistance was determined by the following procedure. A single thallus was impaled with two microelectrodes, one of which served to pass current into the cell while the other recorded the membrane potential. Current pulses were generated by a pulse generator-stimulus isolator, and the resultant changes in the membrane potential were displayed on an oscilloscope screen. Current input and changes in potential were then calculated from photographs of the display and used to construct current-voltage (I-V) curves. Membrane resistance was measured over the range within ± 50 mV of the resting potential.

Analytical methods. Uptake of radioisotopes was monitored by filtering samples on Unipore polycarbonate filters (Bio-Rad), 0.40 μ m pore size. Filters were counted in a scintillation counter (^{14}C , ^3H), or else glued to planchets and counted in a gas-flow counter (^{42}K , ^{86}Rb). The potassium content was measured by flame photometry after extraction with 5% trichloroacetic acid (30 min, 90°C). In some experiments, K^+ uptake was monitored with a K^+ -selective electrode (Beckman no. 39137) connected to an Orion analyzer.

The internal water space was estimated as follows. A concentrated suspension of cells received [^3H]inulin to 0.2 $\mu\text{Ci}/\text{ml}$. Samples were collected by filtration on tared polycarbonate filters, weighed, and then dried over P_2O_5 . The cellular water space is the difference between the total water and the extracellular volume, calculated from the ^3H content. In our hands, this quantity ranged from 1.8 to 3.2 $\mu\text{l}/\text{mg}$ (dry weight) of cells; a value of 2.5 $\mu\text{l}/\text{mg}$ was used for all calculations. Variability of the water space is not surprising since both populations and individual cells differ in the number and size of their vacuoles; old cells are grossly vacuolated. Estimates of internal concentrations must therefore be regarded as approximate. Values for ion flux per unit surface area are equally rough. We took 1 mg (dry weight) of cells to contain 4×10^4 cells, each a sphere 50 μm in diameter; on this basis the surface area of 1 mg of cells is 3.2 cm^2 . This estimate neglects the surface area of the rhizoidal filaments, which is approximately equal to that of the thallus under our growth conditions. The surface area of one organism is thus about $1.6 \times 10^{-4} \text{ cm}^2$, and that of 1 mg of cells is 6.4 cm^2 .

Cytoplasmic pH was calculated from the distribution of [^{14}C]benzoate (3 μM), as described by Maloney et al. (9). The validity of this procedure was confirmed by showing that the distribution is unaffected by the concentration of benzoate between 0.7 and 70 μM and that it responds to alteration of the external pH in the expected manner (data not shown). We would caution readers that the absolute value is subject to error from the variability of the water space, and even more so from the gross inhomogeneity of the cytoplasm: highly acidic vacuoles are common in fungi. Nonetheless, unless *B. emersonii* contains large alkaline compartments that have escaped detection, the distribution of [^{14}C]benzoate should provide at least a first estimate of the cytosolic pH.

Materials. Radioisotopes were purchased from New England Nuclear Corp., and reagents came from standard suppliers (usually Sigma Chemical Co.). Iono-

phores and antibiotics were added as ethanol solutions; sources are listed in previous papers from this laboratory.

RESULTS

The transmembrane potential is a potassium diffusion potential. The magnitude of the membrane potential was markedly dependent on the medium in which the cells were suspended. In the complex medium, PYG potentials varied from -35 to -120 mV; in the defined medium DM2, they varied between -30 and -50 mV; and when cells grown on either medium were transferred to buffered CaCl_2 (SS), the membrane potential immediately rose to -180 mV or even -200 mV. These observations suggested that the main factor governing the membrane potential is the extracellular concentration of K^+ , an inference supported by Fig. 2: the membrane potential varied exponentially as a function of $[\text{K}^+]_0$ over a range from 10 μM to 100 mM K^+ , with a slope of about -50 mV per decade. These particular data were obtained with cells grown on DM2, which was then replaced by buffered CaCl_2 supplemented with various amounts of KCl. Identical results were obtained with cells grown on PYG medium; addition of glucose did not affect the membrane potential.

The intracellular K^+ concentration of cells grown on either DM2 or PYG medium ranged from 0.15 to 0.35 M. The measured membrane potential goes to zero at an external K^+ concentration of 0.1 M, i.e., when the external and internal K^+ concentrations are approximately equal. At no time did we observe a membrane potential more negative than could be accounted for by the K^+ concentration gradients. We therefore conclude that, at least under the conditions of Fig. 2, the membrane potential arises by K^+ diffusion in accordance with the Nernst equation. The deviation from the theoretical slope of -59 mV per decade may reflect the contribution from the diffusion of other ions.

The inset to Fig. 2 illustrates the time course of change in the membrane potential after the addition of K^+ . Within the limits of the technique the transition was abrupt and step-wise, as expected if the potential simply reflects the ratio of potassium ion activities inside the cells and out. There was no sign that the organisms attempted to maintain a negative potential by some active process, such as proton extrusion. If they did, one might expect the potential to rise transiently upon addition of K^+ and then return to a more negative value; this pattern was never seen.

A potassium diffusion potential would be expected to be relatively insensitive to metabolic inhibitors, and in fact neither oligomycin (10 $\mu\text{g}/$

FIG. 2. Dependence of the membrane potential on the extracellular K^+ concentration. *B. emersonii* was grown on DM2 medium. After 16 to 18 h the medium was removed by aspiration and replaced with SS containing varying amounts of KCl. Cells were impaled with electrodes made from either 1.5-mm tubing (○) or 1.2 mm tubing (●). Eight or more cells were impaled for each point, and the results were averaged; bars indicate standard deviation. The slopes correspond to 53 and 47 mV per decade, respectively. Inset: Time-course of the change of membrane potential. A cell in SS plus 0.2 mM KCl was monitored continuously while the medium was successively replaced by SS + 1 mM KCl and SS + 10 mM KCl (arrows). The exchange was completed within 20 to 30 s.

ml) nor NaCN (5 mM) significantly affected the large negative potentials seen at low external K^+ concentration under the conditions of Fig. 2. Both reagents inhibited respiration by over 90% and reduced the cellular ATP content by half. Proton-conducting uncouplers quickly dissipated the membrane potential, perhaps by allowing proton influx to compensate for K^+ efflux. [The compounds used were tetrachlorosalicylanilide (TCS), 1 to 2 μ M, and 2,6-dihydroxy-1,1,1,7,7,7-hexafluoro-2,6-bis (trifluoromethyl)-heptanone-4 (1799), 10 to 20 μ M]. However the membrane potential was also collapsed by a number of other reagents: antimycin (10 μ M), 2-heptyl-4-hydroxyquinoline-*n*-oxide (HOQNO) (10 μ M), valinomycin (4 μ M), and nigericin (2 μ M). We noted that all these inhibitors had drastic effects on the structure of the cytoplasm as observed under the microscope, and we therefore believe that simple explanations based on ion fluxes may not be warranted.

Effect of cations and anions on the membrane potential. To characterize the potassium diffusion pathway, we examined the effect of a series of ions on the membrane potential. Cells were grown in DM2 for 16 h; the growth medium was

then replaced with buffered $CaCl_2$ containing various amounts of the cation (added as the chloride salt), and the membrane potential was determined. In a few cases, the medium was exchanged while the electrode remained inside a cell; the results obtained by the two techniques were the same.

It is clear from Fig. 3 that the cations fall into two groups. Addition of sodium chloride had little effect on the membrane potential until the Na^+ concentration exceeded 10 mM; at lower levels, Na^+ appeared to be far less permeant than K^+ . Other ions that behaved likewise, and are probably impermeant, are Li^+ , Cs^+ , choline, tetraethylammonium ion, and Mg^{2+} . Calcium ions are also quite impermeant by this criterion (see below). The second group consists of ions whose effects on the membrane potential are the same as those of K^+ ; it includes Rb^+ and Tl^+ (up to 1 mM, Tl^+ data not shown). Barium is evidently another permeant cation, though its effects on the membrane potential are more complex than would be expected for a divalent ion that depolarizes with a slope of -29 mV per decade (Ba^{2+} may be expected to block the K^+ channel, but we saw no evidence of this; data

FIG. 3. Effect of various cations on the membrane potential. Cells were grown overnight on DM2, which was then replaced with SS containing the cations (chloride salts). Each point is the average of data from eight or more cells; standard deviations, normally ± 10 mV, were omitted for the sake of clarity. Symbols: (○) Li^+ ; (●) Na^+ ; (△) Rb^+ ; (▲) Cs^+ ; (□) Mg^{2+} ; (■) Ba^{2+} ; (×) choline.

not shown). We conclude that the plasma membrane is permeable to ions whose crystal ionic radius is close to that of K^+ (K^+ , 0.133 nm; Rb^+ , 0.147 nm; Ba^{2+} , 0.153 nm), but impermeable to ions of larger or smaller size (Na^+ , 0.097 nm; Ca^{2+} , 0.118 nm; Cs^+ , 0.167 nm; reference 27). We take this as evidence for a discrete pathway of ion diffusion that selects cations for passage by their size rather than by their valence or chemical properties. The depolarization at high cation concentrations (>10 mM) has a slope greater than 59 mV and may reflect effects of cations on the surface charge rather than the existence of a second, nonspecific channel.

The effect of pH on the membrane potential of cells suspended in buffered CaCl_2 was not pronounced. There was no effect over the range from pH 9 to pH 6; cells at pH 5 were partly depolarized, whereas no potential could be measured at pH 4. Since cells at pH 5 or below leak K^+ and their cytoplasm is visibly disorganized, we conclude only that over the normal range (pH 6 to 9) the plasma membrane is relatively impermeant to protons.

In most of our experiments the only anion added was chloride. However, analogous experiments were done with cells transferred to buffers containing nitrate, maleate, sulfate, or PIPES (1,4-piperazine-diethanesulfonic acid) in various combinations. As shown in Table 1, the measured potential was always the same as that of simultaneous controls containing chloride, suggesting that the potential is not significantly influenced by the external anion. In some experiments we used cells grown in the absence of

chloride; for this purpose DM2 was modified by replacing chloride with nitrate and maleate or with PIPES. This was done to determine whether chloride diffusion contributed to the slope of the plot of $[\text{K}^+]_0$ versus the membrane potential (Fig. 2) and accounts for the deviation from the theoretical value. Cells grown in the absence of chloride were somewhat misshapen but their membrane potential was normal, suggesting that chloride efflux makes little or no contribution to the observed potential.

TABLE 1. Lack of effect of chloride on membrane potential^a

Anions	K^+	Membrane potential (mV)
Maleate, chloride	None ($<10 \mu\text{M}$)	-180
Maleate, nitrate	None ($<10 \mu\text{M}$)	-172
Maleate, chloride	0.1 mM	-155
Maleate, nitrate	0.1 mM	-159
PIPES ^b only	$<10 \mu\text{M}$	-176
PIPES, chloride	0.1 mM	-144
PIPES, sulfate	0.1 mM	-149
PIPES, chloride	1 mM	-114
PIPES, sulfate	1 mM	-113
PIPES, chloride	10 mM	-51
PIPES, sulfate	10 mM	-50

^a Cells were grown in DM2 overnight. The growth medium was then replaced with Tris buffer containing 1 mM Ca^{2+} (as the chloride, nitrate, or PIPES salt). K^+ was added to the concentration shown (as the maleate, chloride, or sulfate). Membrane potentials listed are the average of 7 to 10 individual cells.

^b PIPES, 1,4-Piperazine-diethanesulfonic acid.

FIG. 4. Depolarization of cells by Na⁺ plus K⁺. Cells were grown on DM2 medium, modified to delete chloride. After 16 to 18 h the growth medium was replaced with sporulation solution consisting of 1 mM Tris-maleate and 1 mM Ca(NO₃)₂ (pH 6.8) and the following salts. (A) Sodium added as sodium maleate: (○) no K⁺ added (due to leakage of K⁺ from the cells the external K⁺ level is 10 to 30 μM); (●) 2 mM K⁺ maleate. (B) Sodium added as sodium chloride: (Δ) no K⁺ added; (▲) 1 mM KCl. Bars indicate standard deviation.

Selectivity of the K⁺ channel. To obtain a more quantitative measure of the degree to which the channel prefers K⁺ over Na⁺, the effect of Na⁺ on the membrane potential was determined in the presence of various concentrations of K⁺. The data, a few of which are shown in Fig. 4, were analyzed by assuming that the membrane potential is determined by the passive diffusion of K⁺ and Na⁺ alone, in accordance with the Goldman-Hodgkin-Katz equation. The procedure, outlined in the appendix, assumes that neither chloride nor any other ion contributes to the membrane potential and that the cytoplasmic sodium content, which is not known, remains constant. One can then calculate the ratio of the permeability coefficients, P_{Na^+}/P_{K^+} , from the slope of the linear portion of the plot between 1 and 10 mM Na⁺. The particular data shown here yield P_{Na^+}/P_{K^+} ratios between 0.01 and 0.001. The precision of the calculation is severely limited by the scatter of measurements from individual cells, but warrants the conclusion that P_{K^+} exceeds P_{Na^+} by a factor of at least 100. The conclusion, that the cells are far more permeable to K⁺ than to Na⁺ or to Cl⁻, was reinforced by a series of experiments in which the membrane potential was monitored continuously while the external medium was exchanged for one containing higher (or lower) concentrations of Na⁺ or of Cl⁻ (data not shown). No transient changes in membrane potential were seen, suggesting again that neither Na⁺ nor Cl⁻ movements contribute significantly to the membrane potential.

Measurement of the membrane potential depends on Ca²⁺. The effect of calcium ions on the

membrane potential is shown in Fig. 5. The potential was unaffected by Ca²⁺ concentrations above 1 mM, suggesting that the membrane is quite impermeable to Ca²⁺. Lower concentrations, however, caused progressive depolarization down to -60 mV. Cells in Tris-maleate

FIG. 5. Dependence of the membrane potential on the calcium concentration. Cells were grown on DM2 medium, which was then replaced by Tris-maleate (1 mM, pH 6.8) supplemented with 1 mM ethyleneglycol-bis(β-aminoethylether)-N,N'-tetraacetic acid and CaCl₂ as needed to produce the concentrations of free Ca²⁺ given on the abscissa (calculated by the procedure of Owen, reference 13). (○) Cells in the above media; each point corresponds to 12 to 20 cells. (●) Cells incubated in 1 mM Tris-maleate (pH 6.8) without added Ca²⁺; 1 mM Ca²⁺ was added after 2 h, and the membrane potential was measured 20 min later.

buffer (no added Ca^{2+} ; contamination level 1 to 2 μM) had a membrane potential of about -90 mV. Evidently, free Ca^{2+} ions at a concentration of at least 100 μM are required, either for the membrane to express its selective K^+ permeability or else for the membrane to seal properly around the impaling electrode. The calcium required for display of the normal membrane potential (or for growth) could be replaced by Sr^{2+} , but not by Mg^{2+} , La^{3+} , Tb^{3+} , or Yb^{3+} (data not shown). It is noteworthy that the effects of calcium deprivation were reversible. Even after 2 h in Tris buffer without added Ca^{2+} the normal potential could be restored within minutes by the addition of Ca^{2+} ; cells deprived of Ca^{2+} for 4 h, however, were irreparably damaged.

One possible interpretation of this finding is that, as in many animal cells (11), the K^+ channel itself requires the presence of Ca^{2+} . This does not appear to be the case; removal of calcium ions enhanced both the uptake of $^{86}\text{Rb}^+$ and its efflux, suggesting that the exit pathway is not blocked by calcium depletion (data not shown). Calcium-deficient cells were not obviously permeant to Na^+ or to choline, but were more readily depolarized by lowering the external pH. Subsequently, we found that cells in 1 μM Ca^{2+} had a lowered membrane resistance: 3 to 4 Mohm, compared to 10 to 20 Mohm for normal cells (see below). We therefore suspect that depolarization of the cells at low Ca^{2+} levels results from failure of the membrane to reseal after impalement. All routine measurements of the membrane potential were made in the presence of at least 0.2 mM Ca^{2+} .

Membrane resistance excitability. A current-voltage curve describes the effect of current injection on the membrane potential; it allows one to calculate the membrane resistance and to look for abrupt changes in membrane properties, such as excitability. Figure 6 shows I-V curves for two cells in SS; the input resistance, R_i , was calculated from the linear portion of the curve within ± 50 mV of the resting potential, according to the relationship $R_i = \Delta V / \Delta I$. The average value of R_i from 15 such measurements was 15 ± 5 Mohm. Assuming the surface area of a single cell to be $1.6 \times 10^{-4} \text{ cm}^2$, the specific membrane resistance, R_m , is calculated to be $15 \times 10^6 \text{ ohm} \times 1.6 \times 10^{-4} \text{ cm}^2 = 2,400 \text{ ohm-cm}^2$. This is in the same range as Slayman's published value (17) for *N. crassa*, 5,000 ohm-cm², albeit less than his later estimate that the true value lies closer to 10,000 ohm-cm² (19). It will be obvious that the calculation of R_m depends strongly on the surface area and on the geometry, both of which are particularly uncertain in the case of *Blastocladiella* (Fig. 1).

Over the range of current injection shown in

Fig. 6 the membrane responded monotonically. However, depolarization by about 100 mV produced a significant regenerative response, illustrated in the inset: the membrane potential rose almost to zero and remained there for more than 300 ms after the depolarizing current had ceased. This response, reminiscent of an action potential, indicates that *B. emersonii* is electrically excitable. Studies on the nature of the conductance changes are in progress and will be reported at a future time.

Potassium accumulation and membrane potential in growing cells. In the remainder of this section we shall take it that the membrane potential of *B. emersonii* is largely determined by the K^+ channel, and turn to the question of how the cells accumulate their cytoplasmic K^+ in the first place. Is the cellular K^+ in electrochemical equilibrium with some electrogenic ion pump (a proton pump, or even an anion pump; see Fig. 11A and B) that maintains the cytoplasmic side of the plasma membrane negative, or is K^+ uptake accomplished by a primary active K^+ transport system (see Fig. 11C)?

Figure 7 summarizes a series of measurements made on cells growing in media DMP and PYG, over a range of extracellular K^+ concentrations. The membrane potentials were measured on cells in dish culture after 16 to 18 h of growth. The cellular K^+ content was obtained from parallel suspension cultures (this was necessary in order to obtain enough material for analysis). The cytoplasmic K^+ concentration was always the same, about 0.15 M in these experiments, except at the lowest levels of external K^+ . The graph shows that, in growing cells, the K^+ concentration gradient (the ratio of internal to external K^+) went as high as 3,000, substantially higher than the gradient that could be supported by the membrane potential actually observed. To put it another way, the electrical potential difference that would be in equilibrium with the K^+ concentration gradient, $E_K = (-2.3RT/F) \log ([\text{K}^+]_i / [\text{K}^+]_o)$, is more negative than the observed membrane potential by as much as 50 mV.

Some skepticism is in order with respect to these data, since they are subject to errors of unknown magnitude. The overall cytoplasmic K^+ concentration is known approximately, but the activity at either the inner or the outer face of the plasma membrane is not; an error by a factor of 2 would alter E_K by 18 mV. Likewise, any nonspecific damage or leakiness will make the membrane potential appear less negative than it really is. Nevertheless, these data do suggest that the potassium concentration gradient exceeds the membrane potential.

Two sets of numbers should be singled out for discussion. Cells grown on DMP medium at 50

FIG. 6. Current-voltage plot for two cells of *B. emersonii*. The cells were grown in medium DM2, which was then replaced with SS containing 0.1 mM KCl. Each cell was impaled with two electrodes, one to inject current and the other to monitor the membrane potential. Inset: Effect of current injection on the cell's membrane potential. The lower traces show the amount of current injected, and the upper ones show the resulting deflection of the membrane potential. Note excitable behavior when depolarization of the membrane approached 100 mV. This particular cell had a resting potential of -150 mV.

μM K^+ were clearly K^+ -starved. When a batch of such cells was supplied with small amounts of K^+ the cells accumulated it avidly, raising their internal K^+ concentration from 0.13 to 0.2 M while maintaining the K^+ concentration gradient at about 5,000, or 220 mV. This net K^+ uptake was precisely correlated with net expulsion of protons from the cells, in an amount equivalent to at least half of the K^+ (Fig. 8). Since the measured membrane potential of cells grown in this medium (albeit in dishes rather than in suspension) is only -130 to -160 mV, K^+ accumulation cannot be accounted for by the electrical gradient. By the same token, cells growing in PYG medium at 1.1 mM K^+ con-

tained 0.2 to 0.4 M internal K^+ . The membrane potential of PYG cells was particularly variable, ranging from -35 to -120 mV. The data plotted in Fig. 7, -102 ± 7 mV, were obtained after supplementation of the growth medium with 1 mM CaCl_2 (which, incidentally, suggests once again that insufficiency of Ca^{2+} may give spuriously low potentials). Even so, the measured value fell significantly short of the -140 mV required to account for the K^+ concentration gradient in terms of an equilibrium distribution. We conclude that, in growing cells, K^+ is not distributed across the plasma membrane in accord with a potential established by a proton pump, but is accumulated actively, and also that

FIG. 7. Membrane potential and K^+ concentration in growing cells. Cells were grown overnight in media of various K^+ concentrations. Membrane potentials were obtained by impaling cells grown in dish culture for 16 to 18 h. To measure internal and external K^+ , cells were grown in liquid suspension in batches of the same media. Symbols: ($\circ, \Delta$) membrane potentials, DMP media of varying K^+ concentrations; ($\bullet$) E_K or potassium concentration gradient, DMP media; ($\square$) membrane potential, PYG medium plus 1 mM $CaCl_2$; ($\blacksquare$) E_K , PYG medium.

the membrane potential of growing cells is a little less negative than expected from the K^+ gradient, presumably due to the movement of other ions.

***Blastocladiella* maintains a pH gradient.** Proton movements hold an important place in the ionic relations of fungi. To measure the internal pH we used the distribution of [^{14}C]benzoate, as described in Materials and Methods. As shown in Fig. 9, cells growing in either PYG or DM2 medium maintained an internal pH of nearly 8, for a pH gradient of about 1 unit, interior alkaline. Cells transferred to sporulation solution also kept their cytoplasm alkaline by about a unit for at least 60 min. Note that H^+ , like K^+ , is transported counter to the electrochemical potential gradient.

Maintenance of the pH gradient was dependent on concurrent metabolism as well as membrane integrity. The gradient was abolished by the inhibitors of respiratory ATP generation HOQNO, oligomycin, or NaCN, by the proton-conducting ionophores TCS and 1799, and also by 10 mM sodium azide.

Potassium circulation. We arrive at the hypothesis that the potassium pool of *B. emersonii* reflects a dynamic balance between two (or more) transport pathways: K^+ uptake by some kind of active process, and passive efflux by the K^+ channel. Some characteristics of this balance can be inferred from the exchange of cellular K^+ with $^{42}K^+$. As shown in Fig. 10, turnover of the K^+ pool in cells growing in PYG medium can be interpreted in terms of a single compartment that exchanges with a half-time of

FIG. 8. Net accumulation of K^+ and extrusion of H^+ by potassium-starved cells. *B. emersonii* was grown in liquid suspension on 500 ml of DMP medium containing $50 \mu M K^+$. The cells were allowed to settle and were resuspended in the same medium at one-tenth the original volume. Ten milliliters of suspension was stirred gently while both H^+ and K^+ were monitored with the appropriate electrodes. In the steady state, the external K^+ concentration was $35 \mu M$ (pH 5.90). At the arrow, 2,000 nmol of KCl was added. The cycle of potassium uptake and proton extrusion could be repeated several times before the cells were saturated.

FIG. 9. Intracellular pH of cells in liquid suspension: (A) in PYG medium (pH 7.0); (B) in SS (pH 6.8). Inhibitors were added at the arrow, as follows: (O) none (ethanol only); (●) TCS [$2 \mu\text{M}$ in (A), $0.2 \mu\text{M}$ in (B)]; (Δ) HOQNO, $10 \mu\text{M}$; ($\blacktriangle$) oligomycin, $10 \mu\text{g/ml}$.

30 min. The K^+ content of the cells remained constant for the duration of the experiment ($0.80 \mu\text{mol/mg}$ or 0.36 M); the flux of K^+ through the pool corresponds to about $17 \text{ nmol/min per mg}$

of cells. If one assumes the K^+ flux to be homogeneously distributed over the cell surface, a somewhat unlikely assumption, its magnitude would be $2.7 \text{ nmol/min per cm}^2$. In cells that had been washed and resuspended in SS plus 1 mM KCl turnover was slower, corresponding to a half-time of 40 min and a flux of about 12 nmol of K^+ per min per mg of cells. The rate of $^{42}\text{K}^+$ uptake was a saturable function of the K^+ concentration, with an apparent K_m of $30 \mu\text{M}$ (data not shown).

The initial rate of $^{42}\text{K}^+$ uptake by cells in PYG medium (or in buffer) was inhibited by about 90% in the presence of proton-conducting uncouplers (TCS, 1799) or of inhibitors of respiratory ATP production (HOQNO, oligomycin, NaCN). By contrast, the rate of $^{42}\text{K}^+$ efflux from cells grown overnight in PYG medium, labelled with $^{42}\text{K}^+$, and then suspended in unlabeled PYG was little affected by any of these inhibitors (data not shown). While by no means conclusive, these findings are again consistent with the view that K^+ influx and efflux represent distinct pathways, the former being dependent on metabolism (presumably ATP), and the latter being passive.

FIG. 10. Turnover of the potassium pool. Cells were grown overnight as liquid suspension in PYG medium. To one portion (O), $^{42}\text{K}^+$ was added at 0 min. Samples were withdrawn at intervals and analyzed for both K^+ and $^{42}\text{K}^+$. A second portion (Δ) was filtered to collect the cells; these were then washed on the filter, suspended in SS plus 1.0 mM KCl , and incubated with shaking for 15 min. At this point $^{42}\text{K}^+$ was added (0 min) and samples were withdrawn at intervals thereafter. Results were plotted as a fraction of the pool exchanged at time t , where SA_t is the specific activity of the cellular K^+ at time t , and SA_∞ is the specific activity of the total K^+ in the suspension.

DISCUSSION

In the two decades since Peter Mitchell first formulated the chemiosmotic theory, the role of proton pumps in microbial energy coupling has been generally accepted. Not only bacteria, but also a variety of fungi, algae, and higher plants have been shown to extrude protons across the plasma membrane by means of primary, electrogenic transport systems, and to utilize the electrochemical proton potential (or proton motive force) to perform useful work, particularly the accumulation of ions and nutrients. Proton extrusion in eucaryotic cells has been assigned to electrogenic proton-translocating ATPases, several of which have been characterized (see reviews in references 1, 3, 3a, 14, and 19). It now appears that *B. emersonii* constitutes an exception to this widespread pattern. Our results suggest that this organism (classified as a chytrid, and taxonomically quite remote from the ascomycetes such as yeasts or *Neurospora*) lacks an electrogenic proton pump; instead, the membrane potential arises by diffusion of K^+ out of the cells, down a gradient established by a K^+ pump. If our interpretation is correct we have a novel pattern of ionic currents whose centerpiece is the circulation of K^+ across the plasma membrane.

The heart of the matter is our observation that the membrane potential can be entirely attributed to K^+ diffusion through a relatively specific channel that passes K^+ , Rb^+ , and also Ba^{2+} , but rejects both smaller and larger cations as well as anions (Fig. 2 and 3). The channel selects K^+ over Na^+ by a factor of at least 100 and probably closer to 1,000; in fact, we obtained no evidence for the presence of any other ion channel so long as the membrane was in an unexcited state. The membrane potential varies as a function of the K^+ concentration gradient over four orders of magnitude in accordance with the Nernst equation, and was never seen to exceed the expected value. This should be contrasted with Slayman's measurements on *N. crassa* (17). He, too, noted a partial dependence of the potential on the extracellular K^+ concentration, but K^+ diffusion made only a small contribution: under most conditions the membrane potential was more negative than the K^+ diffusion potential by as much as 80 mV. Thus, whereas Slayman's data provided prima facie support for an electrogenic pump, ours do not.

Slayman and his associates (18, 20) also reported that the membrane potential of *Neurospora* is highly sensitive to various metabolic inhibitors, further evidence for an electrogenic pump. In our hands, the membrane potential was relatively insensitive to NaCN and oligomycin, which inhibit respiration almost completely;

it was depolarized by proton conductors such as TCS, 1799, and carbonyl cyanide *m*-chlorophenylhydrazone. This is what one would expect if the potential were due to K^+ diffusion across a proton-impermeable membrane. However, the effects of ionophores and inhibitors must be interpreted with extreme caution. The structure of eucaryotic cells is complex, and *B. emersonii*, for one, is large enough that one can readily see the havoc that these reagents wreak on cytoplasmic organization. Depolarization could easily result from structural, rather than metabolic, damage. Overall, we cannot claim to have demonstrated the absence of an electrogenic proton pump, only that we have found no evidence for its presence.

Granting that there is a potassium channel that determines the membrane potential by passive conductance, at least under the conditions imposed in Fig. 2 and 3, we must ask how the cells acquired their K^+ pool in the first place. Figure 11 summarizes three possible mechanisms. In both mechanisms A and B, K^+ accumulates in the cell via the K^+ channel, in response to a negative membrane potential maintained by some other ion pump: one that expels H^+ in A, one that takes up chloride ions in B. The latter mechanism can be rejected on the grounds that chloride is not required for growth, for K^+ accumulation, or to establish the normal membrane potential. All mechanisms of this kind can, in principle, be excluded by demonstrating that the cytoplasmic K^+ concentration is too high to be in equilibrium with the membrane potential (Fig. 7 and 8). As noted in Results, neither these data nor the indication that K^+ uptake requires an input of metabolic energy whereas efflux does not, are entirely compelling; they certainly lend no support, however, to the hypothesis that K^+ accumulates in response to a potential generated by another ion pump.

We therefore favor the alternative (Fig. 11C) that K^+ is accumulated by a primary K^+ pump and leaks out through the channel, thereby generating the membrane potential. This scheme is entirely consistent with the experimental observations, though it remains tentative in the absence of inhibitors or mutations that selectively affect either pump or leak. If the membrane potential reflects K^+ diffusion alone, the pump must be electroneutral. The identity of the co- or counterion is not known at this stage. However, keeping in mind that neither Na^+ nor Cl^- is required for growth (26), and also that the cells take up K^+ by exchange for H^+ (Fig. 8) and maintain their cytoplasm alkaline with respect to the medium (Fig. 9), protons are most likely to be the obligatory counterion. We therefore propose that K^+ is accumulated by exchange for protons, presumably driven by ATP (Fig. 11C).

FIG. 11. Possible mechanisms to account for K^+ movements, membrane potential, and transcellular currents in *B. emersonii*. For discussion see text. (A) Proton pump; (B) anion pump; (C) K^+ circulation.

The scheme shows the pump to be electroneutral, but our data do not allow us to assert this with any confidence: given the low membrane resistance, an electrogenic potassium pump would contribute only about 20 mV to the membrane potential, an increment that could have been missed. If the pump does prove to be electroneutral, additional ion fluxes must be expected to preserve overall electroneutrality (and also, to account for the observation that the membrane potential is less negative than E_K ; Fig. 7). These are indicated in the scheme by the entry of protons into the rhizoid, but we would emphasize that the supporting evidence for this assignment (4, 22) remains entirely circumstantial.

Several elements of this hypothesis have appeared in the literature before. Studies with zoospores of *B. emersonii* (5, 26) led to the

recognition of a K^+ -conducting pathway and to the suggestion that the membrane potential is controlled by K^+ diffusion. The notion that K^+ is accumulated by ATP-driven K^+/H^+ exchange has been discussed by plant physiologists, particularly in the electrophysiological context (2, 6, 12, 14, 24, 25). Very recently, Sze (23) presented data suggesting that the ATPase of plant plasma membrane vesicles may catalyze K^+/H^+ exchange, and Malpartida and Serrano (10) made a similar proposal for yeast. ATP-driven K^+/H^+ exchange in gastric mucosa has been thoroughly documented by Sachs and his associates (review in reference 15), but its status in the world of plants and fungi remains that of a working hypothesis.

One of the attractive features of the K^+ circulation (Fig. 11C) is that it can readily account for the transcellular electrical current, of the order of $1 \mu A/cm^2$, reported by Stump et al. (22). The flux through the K^+ pool is substantial: 2.7 nmol of K^+ per min per cm^2 corresponds to a current of $4.5 \mu A/cm^2$ (in *Neurospora*, Slayman [19] estimated 20 to $30 \mu A/cm^2$, carried by protons). If we take the driving force of K^+ efflux, $E_K - V_m$, to be 20 mV (Fig. 7) and the specific membrane resistance, R_m , to be $2,400 \text{ ohm-cm}^2$, the current predicted by Ohm's Law is $I = (20 \times 10^{-3}) / (2.4 \times 10^3) = 8 \mu A/cm^2$; without attributing excessive significance to this calculation, the good agreement between the K^+ flux and the expected current is nevertheless reassuring. Any inequality of the distribution of ion pumps or channels would result in part of this current being carried clear across the cell: perhaps K^+ channels are preferentially localized in the thallus or proton channels in the rhizoid. The latter idea is particularly appealing in view of the evidence that the rhizoid is a major site of nutrient absorption (4; D. L. Kropf, unpublished data) and might be the locus of electrogenic proton-symport carriers. The hypothesis that the outward limb of the transcellular current in vegetative cells (and even in sporulating cells) is carried by K^+ is consistent with all the data now on hand, and particularly with the surprising observation (22; repeated in conjunction with the present work) that the transcellular current requires no extracellular ions except for Ca^{2+} . However, until there is positive evidence to identify the ions that carry current into the rhizoid, further discussion of the genesis and function of the transcellular current does not seem warranted.

APPENDIX

According to the Goldman-Hodgkin-Katz equation,

$$V_m = \frac{RT}{F} \ln \frac{P_K[K^+]_o + P_{Na}[Na^+]_o + P_{Cl}[Cl^-]_i}{P_K[K^+]_i + P_{Na}[Na^+]_i + P_{Cl}[Cl^-]_o}$$

If neither chloride nor any other ion contributes significantly to V_m , the last term can be omitted. Setting $P_{Na^+}/P_{K^+} = \alpha$, then:

$$V_m = \frac{RT}{F} \ln \frac{[K^+]_o + \alpha[Na^+]_o}{[K^+]_i + \alpha[Na^+]_i}$$

By measuring the membrane potential at two concentrations of Na^+ , $[Na^+]_o$ and $[Na^+]'_o$, one obtains two values for the potential, V_m and V'_m , respectively.

Subtracting the two expressions, we get:

$$V_m - V'_m = \frac{RT}{F} \ln \frac{[K^+]_o + \alpha[Na^+]_o}{[K^+]_o + \alpha[Na^+]'_o}$$

By taking antilogs and solving for α , we obtain:

$$\alpha = \frac{[K^+]_o (e^{\beta} - 1)}{[Na^+]_o - [Na^+]'_o (e^{\beta})}$$

where $\beta = (V_m - V'_m)(F/RT)$.

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