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Calcium-Dependent Anion Channel in the Water Mold, *Blastocladiella emersonii*

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Summary. Injection of depolarizing current into vegetative cells of the water mold *Blastocladiella emersonii* elicits a regenerative response that has the electrical characteristics of an action potential. Once they have been taken past a threshold of about −40 mV, cells abruptly depolarize to +20 mV or above; after an interval ranging from several hundred milliseconds to a few seconds, the cells spontaneously return to their resting potential near −100 mV. When the action potential was analyzed with voltage-clamp recording, it proved to be biphasic. The initial phase reflects an influx of calcium ions through voltage-sensitive channels that also carry Sr^{2+} ions. The delayed, and more extended, phase of inward current results from the efflux of chloride and other anions. The anion channels are broadly selective, passing chloride, nitrate, phosphate, acetate, succinate and even PIPES. The anion channels open in response to the entry of calcium ions, but do not recognize Sr^{2+}. Calcium channels, anion channels and calcium-specific receptors that link the two channels appear to form an ensemble whose physiological function is not known. Action potentials rarely occur spontaneously but can be elicited by osmotic downshock, suggesting that the ion channels may be involved in the regulation of turgor.

Key Words action potential · Ca channel · anion channel · fungi · voltage clamp · turgor

Introduction

Our conception of ion movements across the plasma membrane of fungi stems almost entirely from the pioneering research of C.L. Slayman with the filamentous ascomycete *Neurospora crassa* (Slayman, 1965*a,b*; Slayman et al., 1973; Warncke & Slayman, 1980). In this organism, the membrane potential is largely generated by an electrogenic proton pump that couples the hydrolysis of ATP to the extrusion of one or two protons from the cytoplasm. Protons complete the circuit by diverse "leak" pathways, including transport carriers that mediate the symport of protons with glucose and other metabolites (Slayman & Slayman, 1974; Goffeau & Slayman, 1981; Sanders et al., 1983). Similar patterns of ion flow and energy coupling have been observed in yeast, green algae and in higher plants. In all these organisms, work is performed by a current of protons across the plasma membrane that is driven by an electrogenic, proton-translocating ATPase (Poole, 1978; Spanswick, 1981; Harold, 1982).

Blastocladiella emersonii is a water mold, classified by Margulis and Schwartz (1982) in the phylum *Chytridiomycota*. Members of this group have been traditionally placed with the fungi, but are very different from the familiar ascomycetes in appearance, life cycle and also in membrane properties. We have previously reported (Van Brunt et al., 1982) that the plasma membrane of *Blastocladiella* responds as though it were permeable only to K^+. Over a wide range of K^+ concentrations the membrane potential was very close to the potassium equilibrium potential; there was no sign of an electrogenic component, suggesting the absence of an electrogenic proton pump. We now report a second unexpected feature of the *Blastocladiella* plasma membrane, namely, a regenerative depolarization that has the hallmarks of a calcium action potential.

Calcium channels and calcium action potentials exist in a wide variety of plant and animal cells. The properties of calcium channels are equally diverse (Hagiwara & Byerly, 1981; Tsien, 1983) and they support a variety of functions including the excitation of certain neurons and the exocytosis of vesicles during growth or secretion. One consequence of calcium entry is the opening of additional ionic channels; an example of this is the well-characterized calcium-activated potassium conductance (Meech, 1978). Less attention has been paid to calcium-controlled anion channels which occur in various animal cells (Robinson, 1979; Bader et al., 1982; Grinstein et al., 1982; Miledi, 1982; Barish, 1983;

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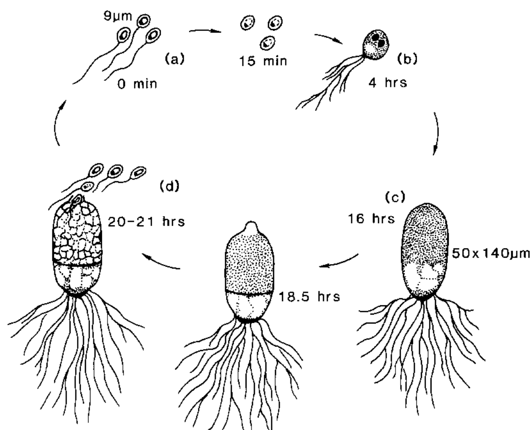

Fig. 1. Life cycle of *Blastocladiella emersonii*. Zoospores (*a*) are motile. When conditions are suitable for growth the zoospores settle down, retract their flagella and sprout rhizoids (*b*). The germlings grow into mature vegetative organisms (*c*). In time these sporulate (*d*), to start the cycle anew

Miledi & Parker, 1984; Owen et al., 1984) and in plants as well (Findlay & Hope, 1964; Lunevsky et al., 1983).

Our findings indicate that in *B. emersonii* Ca^{2+} influx triggers the opening of an anion channel, allowing the exit of chloride and of other anions concentrated within the cell. The efflux of these anions is responsible for the long duration of the action potential. Voltage-clamp analysis of the ionic specificity of this channel indicates little selectivity on the basis of either size or the number of negative charges. The function of this calcium-activated anion channel is unknown; we speculate that the ensemble as a whole participates in the exocytosis of cell wall and plasma membrane precursor vesicles.

Materials and Methods

Organism and Media

B. emersonii begins its life cycle as a flagellated zoospore (Lovett, 1975). The zoospore settles down, germinates and grows into a vegetative organism which consists of a roughly spherical, coenocytic thallus (diameter about 50 μm) and a system of rhizoidal filaments (Fig. 1). The latter serves as a holdfast and has also been implicated in the transport of nutrients to the thallus (Harold & Harold, 1980; Kropf & Harold, 1982). Measurements were routinely made on mature vegetative cells after 17 to 20 hr of growth.

Cells were grown at 24°C on one of two media. i) The complex medium PYG contains (per liter) 1.25 g peptone, 1.25 g yeast extract, and 3 g glucose. The free calcium content is 30 μM, insufficient to support action potentials; therefore in many experiments PYG medium was supplemented with 1 mM $CaCl_2$. ii) The defined medium DM2 contains (in mM) 25 K^+, 12 Na^+, 10 Mg^{2+}, 1 Ca^{2+}, 37 Cl^-, glucose, phosphate, amino acids, trace metals and thiamine (Selitrennikoff & Sonneborn, 1977). Medium DM2 was modified for particular experiments: strontium was substituted for calcium, chloride was omitted or replaced by an equivalent concentration of PIPES, or both substitutions were performed.

Replacement of growth medium by buffered calcium chloride (1 mM $CaCl_2$, plus 1 mM Tris maleate or 1 mM Ca PIPES, both at pH 6.8) induces the cells to sporulate, a process which requires 5 to 6 hr at 24°C. Unless otherwise noted, the measurements reported here were made prior to the appearance of a baseplate, which signals the transformation of the vegetative thallus into a sporangium (*see* Fig. 1).

Electrical Measurements

Cells were grown attached to the bottom of plastic tissue culture dishes (Falcon, 60 × 15 mm). Each dish contained a total of 2.5 × 10^4 cells in 4 ml of medium. Microelectrodes were pulled from borosilicate Omega Dot capillary tubing (1.2 mm OD, 0.9 mm ID; Frederick Haer and Co., Brunswick Maine); the voltage and current-passing electrodes had typical resistances of 20 and 10 MΩ, respectively. Electrodes were filled with 1 M potassium citrate (Slayman, 1965*b*). Ag/AgCl pellets served as reference electrodes; one electrode acted as a virtual ground and the second electrode compensated for any polarization potential while current was flowing. The pellets were immersed in a bath of 1 M potassium citrate and KCl which was connected to the experimental chamber by a 2% agar bridge containing either the medium bathing the cells or, in the case of anion replacement, 10 mM NaCl and 1 mM Ca PIPES. Tip potentials, estimated by measuring the effect of breaking the microelectrode tip at the end of the experiment, never exceeded 5 mV and were ignored.

Cells were routinely impaled with two microelectrodes, with the aid of a pair of "aus Jena" micromanipulators (Jena, German Democratic Republic). The voltage electrode was connected to a KS-700 Dual Microprobe Electrometer (WPI Instruments); current pulses were generated with a pulse generator/stimulus isolator system (WPI Instruments). Current and voltage records were displayed on an oscilloscope screen and were stored in one of two ways. In some experiments, a polaroid picture was taken of the voltage and current traces on a storage oscilloscope; alternatively data points were digitized by an A/D converter and stored on a DEC LSI 11/03 computer. By convention, outward ionic currents are designated as positive (cations out or anions in); inward ionic currents are designated as negative (cations in or anions out).

Two-electrode voltage-clamp experiments were performed by means of a conventional voltage-clamp amplifier. The spatial control of the membrane potential was an important consideration due to the complicated cell geometry. The thallus, where the electrodes are inserted, is roughly spherical and its membrane potential is likely to be adequately controlled. The long, thin rhizoids (*see* Fig. 1), however, are almost certainly not under voltage control; these rhizoids are approximately 1 μm in diameter and have a total surface area equal to that of the thallus. The lack of control over the rhizoids raises the possibility that the voltage-clamp currents are contaminated by contributions from this unclamped membrane. By growing the cells on nucleopore filters such that the thalli are on one side and the rhizoids on the other, and then immersing the rhizoids in mineral oil (Kropf & Harold, 1982), we obtained some evidence that the calcium and anion channels are present in the thallus; but we cannot rule out their presence in the rhizoids as well. The smooth

rise and decay of the early and late currents reinforces our belief that the channels are located largely in the membrane under voltage control.

The resistance in series with the membrane can introduce a difference between the applied potential and that which actually exists across the membrane. The series resistance was measured in the current-clamp mode by injecting a current step and recording the immediate voltage response. The series resistance was 10 kΩ which is small compared to the input resistance (30 to 100 MΩ). The maximum difference between the recorded and true membrane potential is $I_m \cdot R_s$ where I_m is the maximum membrane current (usually not more than 100 nA). This would introduce an error of less than 1 mV and would not affect any of our conclusions.

Leakage current was measured prior to each voltage step by applying a short, small, depolarizing step. The resultant current was then scaled for the voltage step applied, assuming linearity. Calcium and anion currents were not seen in the presence of La^{3+}, and the currents measured in La^{3+} were linear for depolarizing voltage steps from −100 to +20 mV, which indicates that the assumption of linearity is valid over this range. The leakage current for a 100-mV depolarization was about 1 or 2 nA, which is a small fraction of the calcium and anion current.

It was not possible to apply a rapid succession of voltage-clamp steps or to induce action potentials at high frequency. Full recovery from a single action potential or voltage step required about 30 sec. Measurements of reversal potentials and current-voltage curves were therefore made at intervals of 30 to 60 sec.

Reversal potential measurements were made only in those cases in which a reversal of the current could be observed, rather than attempting to extrapolate to zero current. Reversal potentials were determined using the tail current technique (Hodgkin & Huxley, 1952*a*). The relative permeability of the anion channel to different anions was determined by measuring the change in reversal potential, ΔE_{rev} (with the tail current applied at the peak of the anion current) when all the external Cl^- was replaced by another anion *A*. For monovalent anions the relative permeability is obtained from the following equation (Dwyer et al., 1980):

$$\Delta E_{rev} = E_{rev,Cl} - E_{rev,A} = \frac{RT}{zF} \ln \frac{P_{CL}[Cl]_o}{P_A[A]_o}. \quad (1)$$

For divalent anions the equation below was used (Spangler, 1972):

$$\Delta E_{rev} = -\frac{RT}{F} \ln \left(\frac{y}{y'}\right) \quad (2)$$

where

$$y = \frac{-b + \sqrt{b^2 - 4ac}}{2a} \text{ and } y' = \frac{-b' + \sqrt{b'^2 - 4a'c'}}{2a'}$$

$$a = P_{Cl}[Cl]_i \qquad a' = P_{Cl}[Cl]_i$$
$$b = P_{Cl}([Cl]_i - [Cl]_o) \qquad b' = P_{Cl}[Cl]_i$$
$$c = -P_{Cl}[Cl]_o \qquad c' = -4P_A[A]_o.$$

Primed symbols refer to the situation with all external chloride replaced by an equal concentration of anions. Osmotic strength was kept constant with sucrose. We have assumed that the channel is permeable only to anions and that the only internal anion which is both permeant and highly concentrated is chloride.

When chloride was replaced by other anions, a junction potential developed at the agar bridge. This junction potential can be as large as −10 mV. These junction potentials were measured relative to a saturated Ag/AgCl reference electrode and the holding potential was shifted for each substitute anion to maintain a constant membrane potential.

Perfusion Chamber

To replace one medium with another while an experiment was in progress, a perfusion chamber was created within the tissue culture dish containing cells. This was accomplished by placing in the dish Plexiglas® inserts shaped like half-moons, so as to leave a narrow channel in the center. The inserts were lightly coated with stopcock grease in order to minimize the volume of fluid trapped beneath.

Efflux of ^{36}Cl

Cells were grown in modified DM2 medium containing 5 mM ^{36}Cl (2×10^5 cells per dish, 10 ml medium, 75 nCi/ml). They were then incubated for 90 min with fresh radioactive medium containing 0.1 M sucrose; and washed twice with nonradioactive medium (sucrose present) in order to remove extracellular ^{36}Cl. Fresh nonradioactive media of various compositions were then added; samples of the medium were withdrawn at intervals and the amount of ^{36}Cl released from the cells was determined.

Results

Description of the Action Potential

A microelectrode, inserted into a vegetative cell of *B. emersonii* immersed in buffer containing 1 mM $CaCl_2$ and 1 mM KCl, records a membrane potential of about −120 mV (Van Brunt et al., 1982). Constant current pulses were injected through a second microelectrode inserted into the same cell (Fig. 2), so as to alter the membrane potential. As shown in Fig. 3, depolarization was passive as long as the membrane potential remained more negative than about −45 mV. Depolarization beyond this value induced a regenerative, all-or-none response: the cell abruptly depolarized to +20 to +40 mV and spontaneously repolarized after several hundred milliseconds. This response could be reproduced many times with little change in its amplitude, duration or shape. Since the response has many of the electrical characteristics of the nerve action potential we shall refer to it by that name, but do not wish to imply that the action potential of *Blastocladiella* has the same function as that of nerves.

Figure 3 illustrates many of the basic features of the *Blastocladiella* action potential. Once the membrane reached the threshold voltage, it depolarized in an all-or-none manner. The threshold varied from

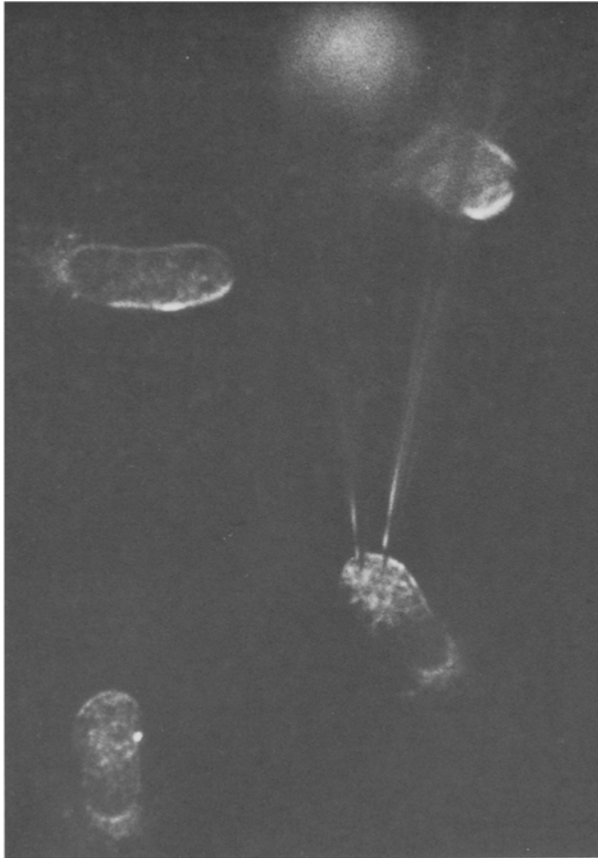

Fig. 2. A vegetative cell of *Blastocladiella* impaled with two microelectrodes

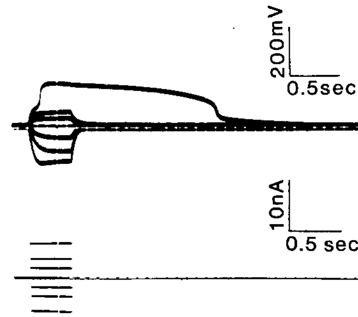

Fig. 3. The action potential. Cells were grown on DM2 overnight; the medium was then replaced by buffered $CaCl_2$ and two microelectrodes were inserted into one cell. One microelectrode was used to inject current, the other monitored the membrane potential (resting potential, −144 mV). The upper set of traces records changes in membrane potential elicited by injection of varying amounts of depolarizing or hyperpolarizing current (lower set of traces). In this instance, injection of 7 nA elicited an action potential with a plateau of +36 mV and a duration of over 1 sec

one cell to another, from −29 to −59 mV. Full recovery from an action potential required about 30 sec. In *Blastocladiella,* as in nerve cells, slow depolarization of the membrane did not elicit an action potential ("accommodation"). Finally, these cells exhibit a feature termed "anode break" (Hodgkin & Huxley, 1952*b*). This response was observed when cells were transiently hyperpolarized and the hyperpolarizing current was then turned off; in some cases the cell, after returning to the resting potential, continued to depolarize and produced an action potential.

Conductance Changes

In order to determine how the membrane conductance changes during an action potential, a train of small, constant-current pulses was injected continuously before, during and after the pulse that triggers the action potential. As shown in Fig. 4 the membrane conductance increased dramatically for the duration of an action potential and then recovered over a period of several seconds. The increased conductance implies that the action potential results from the transient opening of ionic channels.

The Action Potential Requires Ca^{2+} Ions

In principle, the action potential may result from an influx of cations, an efflux of anions, or from a combination of both. As will be shown below, both Ca^{2+} entry and anion exit contribute to the phenomenon.

Omission of Ca^{2+} ions from the medium abolished the action potential. The hypothesis that Ca^{2+} carries at least part of the current during the action potential was reinforced by the finding that the height of the action potential increased as a function of the external calcium concentration. The increase in amplitude varied between cells from 13 to 30 mV per 10-fold change in the calcium concentration (over the range of $[Ca]_o$ from 30 μM to 1 mM).

Neither verapamil (120 μM) nor D-600 (100 μM), reagents known to block calcium fluxes in some animal cells, affected the action potential (nor did tetrodotoxin, which blocks sodium channels in nerve and muscle). Persuasive evidence for a calcium current came, however, from studies on the effects of divalent cations summarized in Table 1, and from voltage-clamp measurements described in the following section. Cobalt, cadmium, lanthanum and several other lanthanides blocked the action potential, with La^{3+} being the most effective. Aside from Ca^{2+}, only strontium ions supported an action potential; Ba^{2+} and Mn^{2+} ions were marginally effective. The characteristics of the action potential var-

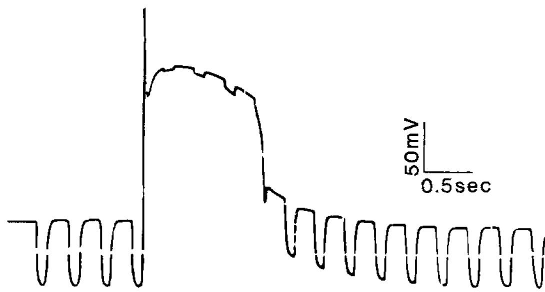

Fig. 4. Membrane conductance increases during an action potential. The experimental conditions were the same as described in Fig. 3, except that the resting potential was −115 mV. A train of hyperpolarizing current pulses, duration 100 msec, was continually applied before, during and after the action potential to monitor changes in membrane conductance. The conductance increased more than 20-fold during the action potential

Table 1. Effect of cations on the action potential[a]

Cation	Action potential in absence of Ca^{2+} (mM)	Inhibition of action potential, with 1 mM Ca^{2+} (mM)
Sodium	No (20)	No (20)
Magnesium	No (20)	No (20)
Strontium	Yes (0.1)	No (5)
Barium	Poor (1)	No (1)
Zinc	No (1)	Yes (10)
Cadmium	No (1)	Yes (0.5)
Manganese	Poor (1)	Yes (1)
Cobalt	No (1)	Yes (>0.2)
Nickel	No (1)	No (1)
Lanthanum	No (1)	Yes (>0.03)

[a] In addition to the ions listed, the following neither substituted for Ca^{2+} nor inhibited: K^+ (100 mM), Cs^+ (2 mM), tetraethylammonium (10 mM).

ied somewhat with the ion: the threshold, whose mean value was about −45 mV with Ca^{2+}, fell to −73 mV when Sr^{2+} was provided instead, and trains of spontaneous action potentials were occasionally seen. Since several well-documented calcium channels pass Sr^{2+} and Ba^{2+} and are blocked by lanthanides (Hagiwara & Byerly, 1981), we conclude that the action potential of *B. emersonii* requires the transient opening of calcium channels.

What other cations could contribute to the action potential? The only other positively charged ions that can enter the cell are K^+, Tris and H^+. Omission of the Tris maleate buffer had no effect upon the action potential. Most of our experiments were carried out at pH 6.8; since the cytoplasmic pH is about 7.8 (Van Brunt et al., 1982), proton influx could possibly contribute to the action potential. This hypothesis is discounted by the observation that the action potential was the same over an external pH range from 5 to 9 (at pH 9, proton influx could depolarize the membrane only to −60 mV, whereas the action potential reached +30 mV). Potassium ions are ruled out by the fact that K^+ is already at electrochemical equilibrium (Van Brunt et al., 1982); moreover, omission of K^+ from the medium had no effect on the action potential.

Anion efflux, chloride in particular, must also be considered. The chloride content of *B. emersonii* is 36 mM, that of PYG medium is 3 mM; E_{Cl} in PYG medium is thus approximately +60 mV. Since the peak of the action potential was usually between +20 and +40 mV, the permeant ion might be chloride. The possibility of a chloride action potential was initially rejected because cells grown on chloride-free medium (with PIPES replacing chloride) generate normal action potentials in the absence of either internal or external chloride (*data not shown*). Nevertheless, as will be shown below, chloride is one of a broad class of anions that flow out of the cell during an action potential.

Biphasic Current Flow during the Action Potential

That the phenomenon under study here is not a pure Ca^{2+} action potential became apparent when the flow of current was examined under voltage clamp. Currents measured under voltage clamp (Fig. 5*A*) are compared with the action potential (Fig. 5*B*) for the same cell with either Ca^{2+} or Sr^{2+} as the external divalent cation. Figure 5*A* (solid line) depicts a cell whose resting potential was first held at −102 mV and then abruptly clamped at −22 mV in the presence of Ca^{2+}. The time-course of current flow was clearly biphasic, composed of an initial inward current followed by a late, prolonged and extensive phase of inward current. The peak current registered in this experiment was about 55 nA; if all the inward current represented Ca^{2+} ions, 0.2 peq Ca^{2+} would have entered the cell, raising its intracellular Ca^{2+} content by an implausible 3 mM.

In fact, it appears that the two phases of current flow correspond to distinct ionic fluxes. The early phase is certainly carried by Ca^{2+} ions. It required the presence of either external Ca^{2+} or Sr^{2+}, the current was blocked by 50 μM La^{3+} and its apparent reversal potential was strongly positive (+36 mV in Fig. 6, +91 mV in a second cell by use of the tail current technique described below). The larger, delayed current flow represents an efflux of anions that will be discussed below. Remarkably, quite another time course was observed when Sr^{2+} or Ba^{2+}

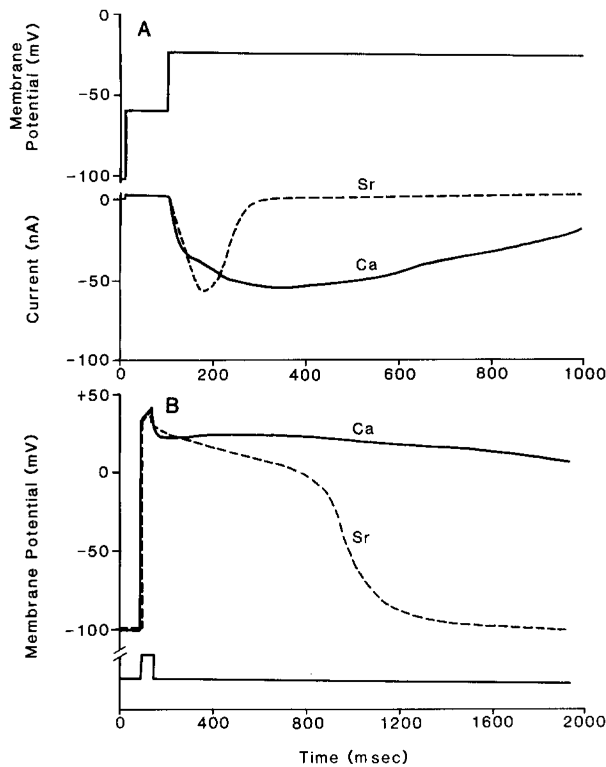

Fig. 5. Voltage-clamp current and action potentials supported by calcium and strontium ions. *A*. Biphasic current. Solid line: Cells were grown on DM2; the medium was replaced with buffered 1 mM $CaCl_2$, which flowed through the culture chamber for the duration of the experiment. A cell was impaled and clamped to a holding potential of −102 mV (upper trace). The potential was then stepped to −62 mV for 100 msec to measure the leak current; the voltage was then clamped to −22 mV, initiating inward current flow. The solid line marked Ca shows the biphasic time course of current flow. Dashed line: the same cell, but the chamber was perfused with buffered 1 mM $SrCl_2$; under these conditions, only the initial phase of the inward current is evident. *B*. Action potentials recorded from a single cell (same cell as *A*) in the presence of either Ca^{2+} (solid line) or Sr^{2+} (dashed line) ions. The membrane potential was held at −102 mV prior to the action potential. Injected current (10 nA) indicated beneath the action potentials. In the presence of $CaCl_2$, the duration of the action potential was greater than 4 sec; in the presence of $SrCl_2$, it was less than 1 sec

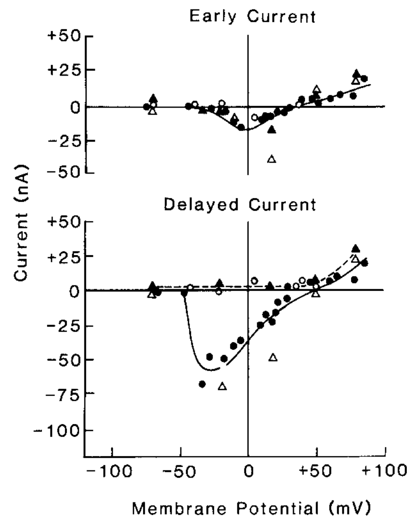

Fig. 6. External calcium ions are necessary and sufficient to trigger the delayed current. Cells were grown on DM2 containing either $CaCl_2$ or $SrCl_2$; the medium was then exchanged for buffered $CaCl_2$ or $SrCl_2$, as indicated by the symbols. Cells were clamped to a holding potential of −100 mV and then stepped to potentials ranging from −70 to +100 mV. For each potential we determined both the early current (measured 20 msec after the voltage step) and the maximal delayed current. In this experiment, leak currents were not subtracted. Symbols: (●), Ca^{2+}-grown cells in $CaCl_2$; (○), Ca^{2+}-grown cells in $SrCl_2$; (△), Sr^{2+}-grown cells in $CaCl_2$; (▲), Sr^{2+}-grown cells in $SrCl_2$

was substituted for Ca^{2+}. As shown for Sr^{2+} in Fig. 5*A* (dashed line), there was a brief influx of current but this was not followed by the larger delayed current. The reversal potential with Sr^{2+} was +68 ± 24 mV (mean ± standard deviation, n = 11). The difference between these two ions is also reflected in the time course of the action potential, which tends to be significantly shorter with Sr^{2+} than with Ca^{2+} (Fig. 5*B*).

That calcium ions trigger the delayed current, but strontium ions do not, is documented by the current-voltage curves in Fig. 6. Cells were grown on DM2 medium containing either 1 mM Ca^{2+} or 1 mM Sr^{2+}, put into either buffered 1 mM $CaCl_2$ or buffered 1 mM $SrCl_2$, and subjected to different depolarizations. The pattern of early current was the same whether the cells had been grown on Ca^{2+} or on Sr^{2+}, and was also the same whichever ion was present in the external medium (Fig. 6*A*). The results for the late current were different: regardless of how the cells were grown, and thus regardless of the internal divalent cation, only when Ca^{2+} ions were present externally did the late current ensue.

We conclude that depolarization of the plasma membrane elicits the opening of channels that allow the passage of Ca^{2+}, Sr^{2+} and also Ba^{2+} ions (Mn^{2+} ions, which did support a small regenerative response, were not studied under voltage clamp). The entry of Ca^{2+} ions, but not of Sr^{2+} or Ba^{2+}, triggers the opening of a separate set of channels which, as will be shown next, carry chloride and other anions out of the cell.

Chloride Efflux Carries the Delayed Current

Evidence that the two phases of current flow are carried by different ions is presented in Fig. 7. Cur-

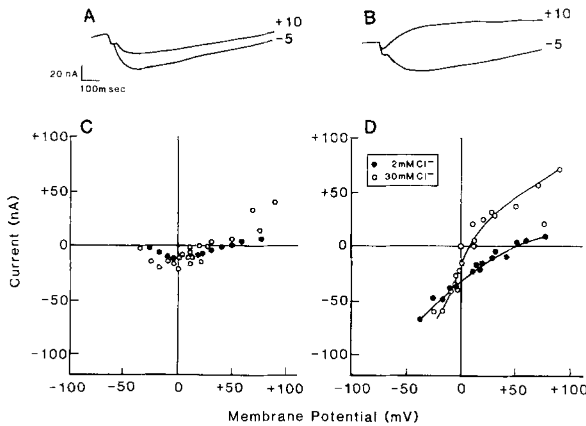

Fig. 7. Effect of external chloride upon current. All records are from a single cell grown in DM2. Holding potential: −100 mV. Voltage-clamp current was recorded either (*A*) in 2 mM $[Cl]_o$ (1 mM $CaCl_2$, 1 mM Tris maleate) or (*B*) in 30 mM $[Cl]_o$ (30 mM choline Cl and 1 mM Ca PIPES). The potential to which the membrane was clamped is indicated beside the current records. Current-voltage curves were plotted for the early current (20 msec after the voltage shift) in *C* and for the peak of the late current in *D*. The change in external chloride had little effect upon the early current but shifted the reversal potential of the late current from +48 to +6 mV

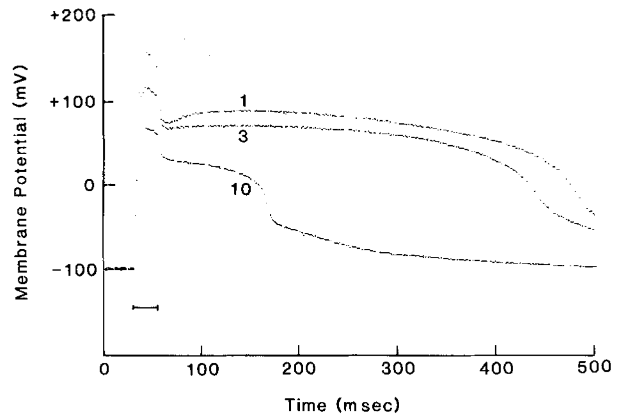

Fig. 8. Effect of external Cl^- upon the plateau of the action potential. Holding potential = −100 mV. Injection of current (bar) initiated action potentials. The external chloride was varied, beginning with 10 mM and successively decreasing to 3 mM and then to 1 mM

rent-voltage curves similar to those shown in Fig. 6 were generated for different external concentrations of chloride. Some of the voltage-clamp currents are shown for low chloride (2 mM, Fig. 7*A*) and high chloride (30 mM, Fig. 7*B*). The delayed currents are obviously different at the more positive potentials. The peak early current, measured 20 msec after the start of the clamp, is plotted in Fig. 7*C*; these curves show little difference. The reversal potential was shifted from +44 to +30 mV, but this is probably due to the fact that nearly a third of the current measured at 20 msec passes through the delayed (anion) channel (*see* Fig. 10). The delayed current, measured at its peak, is plotted in Fig. 7*D*; the reversal potential for high external Cl^- is shifted by 42 mV relative to that for low external Cl^-. These experiments indicate that the delayed current goes through a channel that also passes chloride, while the early current has little or no chloride component.

Changing the external chloride concentration should have an effect upon the late, plateau phase of the action potential. As shown in Fig. 8, the height of the action potential shifted by 62 mV for a 10-fold change in [Cl] (from 10 to 1 mM).

If chloride carries the delayed current, the reversal potential of the delayed current should be governed by the electrochemical potential of chloride and should vary as a function of the chloride concentration gradient. An experiment with cells grown on DM2 medium is shown in Fig. 9. We measured "tail currents," elicited at the peak of the delayed current, to test the relationship between $E_{reversal}$ and $[Cl^-]_o$. The inward current was triggered as usual by shifting E_m, in this case from −90 to −16 mV. At the height of the response (about 200 msec), E_m was shifted to a different value; the resulting tail current represents the flow of ions in response to the new potential gradient. In the present instance, the direction of current flow turned outward when E_m was +40 mV. Figure 9*B* summarizes a series of such experiments over a range of external chloride concentrations. When the external chloride concentration was changed from 2 to 30 mM the delayed current varied with E_{Cl} in the expected manner according to the Nernst relationship (a slope of 55 mV).

The only cations, other than calcium, that could contribute to the delayed current are H^+ and K^+. The effect of changing the pH from 5.5 to 9 (the cell would not tolerate more extreme pH's) upon the reversal potential of the delayed current was measured. The reversal potential shifted only 12 mV (more positive) for a 3000-fold increase in external hydrogen ion concentration. Variation in external $[K^+]$ did affect the reversal potential at the time of peak inward current, but the effect was small (a 5-mV positive shift for a 10-fold decrease in $[K^+]_o$) and was in the opposite direction from that expected for a K^+ contribution to the late current.

Additional evidence that the delayed current is carried by anions came from the use of ethacrynic acid. Ethacrynic acid blocks anion channels in algae (Lunevsky et al., 1983) and in red blood cells (Motais & Cousin, 1976). Ethacrynic acid blocked the delayed current at 0.5 mM (Fig. 10): the remaining

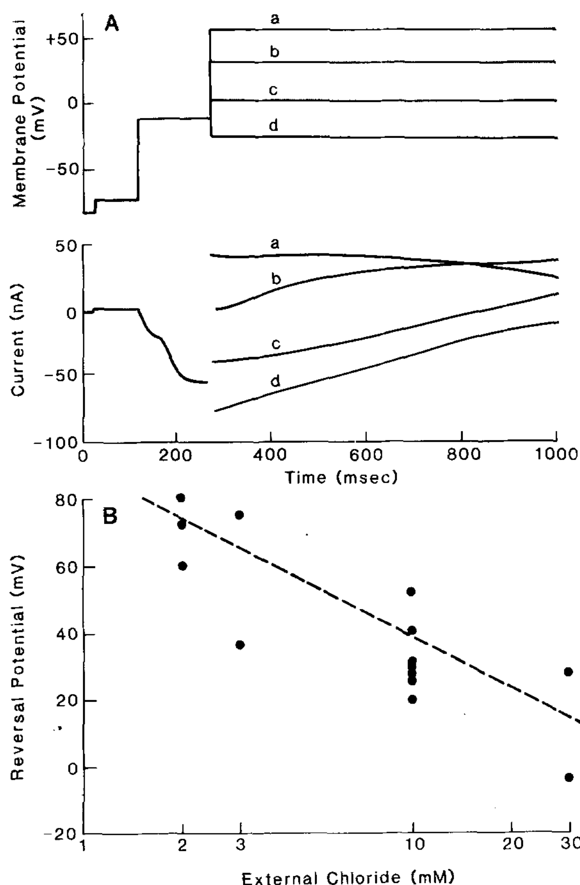

Fig. 9. *A*. Tail currents. Cells were grown in DM2, which was then replaced with 1 mM Ca PIPES. A vegetative cell was impaled and the buffer replaced by 1 mM Ca PIPES containing 10 mM sodium chloride. The cell was clamped to a holding potential of −85 mV, and subsequently stepped to −75 mV for 60 msec to measure the leak current. The potential was then stepped to −12 mV, initiating an inward current (t = 115 msec). At the peak of current flow, t = 265 msec, a series of tail currents was generated in order to determine the reversal potential. Letters identify corresponding current and voltage traces. *B*. Reversal of the delayed current as a function of external chloride: pooled data from a series of experiments analogous to *A*. The dashed line shows the relationship expected if chloride ions were the only charge-carrying species

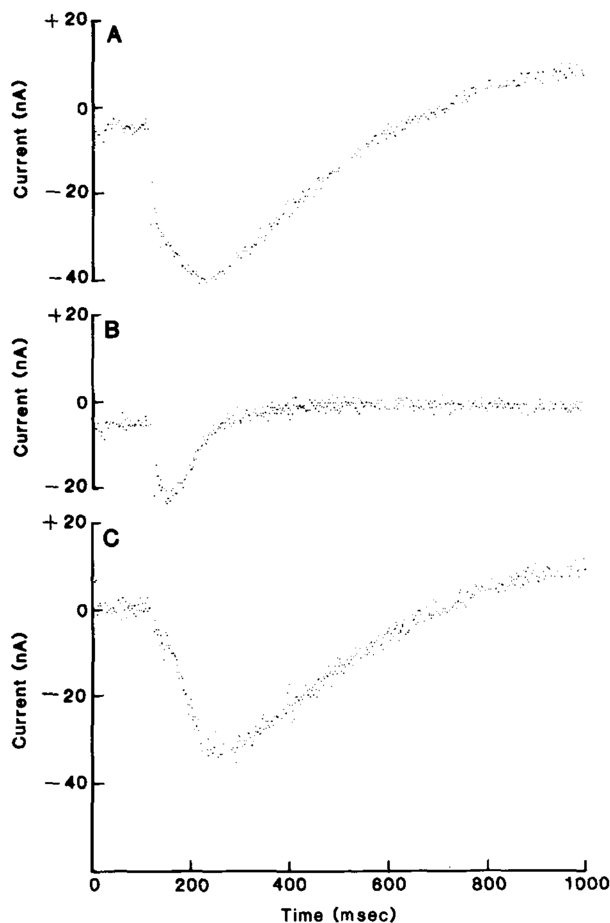

Fig. 10. Effect of ethacrynic acid upon the voltage-clamp current. Cells were grown in DM2, which was then replaced by 1 mM $CaCl_2$, 1 mM Tris maleate, pH 6.8. The holding potential was −100 mV; to initiate current flow the membrane potential was shifted to +12 mV. *A*. Control. *B*. Inward current observed 1 min after the addition of 1 mM ethacrynic acid to the medium. *C*. The difference between currents in *A* and *B*. This is assumed to be the Ca-activated anion current

current, which we assume to be carried by Ca^{2+}, appears very similar to the current seen when all the Ca^{2+} was replaced by Sr^{2+}. Ethacrynic acid had no effect upon Sr^{2+} currents (*not shown*). The use of ethacrynic acid allows us to separate the divalent and anion currents. The delayed, anion current (obtained by subtracting the Ca^{2+} current measured in ethacrynic acid from the total current) is plotted in Fig. 10*C*. Since the early and delayed currents overlap in time, measurements of the early current (20 msec after the voltage step) are somewhat contaminated by anion current. This problem also exists to a lesser degree for measurements of the delayed current (150 msec after the voltage step). Assuming that ethacrynic acid blocks the anion channel without altering the calcium channel, we calculate from Fig. 10 that 29% of the current measured at 20 msec actually comes from the anion channel, while 14% of the current at 150 msec is carried by Ca^{2+}.

Several Anions Contribute to the Delayed Current

Blastocladiella grows indefinitely in the absence of chloride, and the cells generate normal action potentials consisting of an initial calcium entry followed by delayed anion efflux (*data not shown*). The anion in this case cannot be chloride; either one

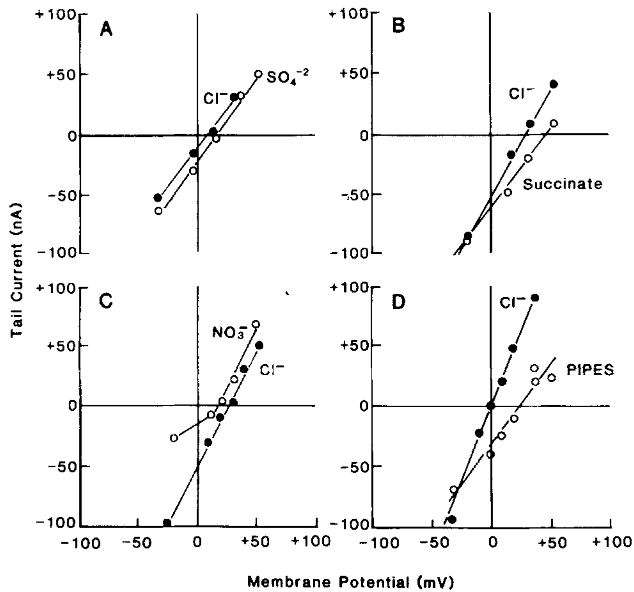

Fig. 11. Specificity of the anion channel. Cells were grown in DM2, which was replaced by 1 mM Ca PIPES plus 10 mM sucrose. After a cell was impaled the buffer was replaced by one containing 1 mM Ca PIPES, the sodium salt of the anion under investigation, and sufficient sucrose to maintain a constant osmotic pressure. In each case, a series of tail currents was generated as illustrated in Fig. 9. (Anions more permeant than chloride have reversal potentials more negative than that of chloride, less permeant ones have more positive reversal potentials.) Curves are shown for chloride, sulfate, succinate, nitrate and PIPES. Sets *A*, *B*, *C* and *D* depict different cells

or more cellular anions take its place, or else the permeant anion must be introduced by the intracellular electrodes. The data suggest that the channel permits the passage of a range of anions including PIPES.

In order to discover which ions move through the Ca^{2+}-activated channel, a series of tail-current experiments analogous to Fig. 8 was carried out and the results were combined to generate a set of current-voltage curves for the delayed current (Fig. 11). Cells grown in DM2 were examined in the presence of 10 mM anions as indicated. The essential results are embodied in the sequence of reversal potentials shown in Table 2. These were calculated as described in Materials and Methods. This sequence reflects the relative permeability of the membrane to anions: nitrate > chloride > PIPES > sulfate > succinate. Phosphate, acetate and malate are also permeant, but the permeabilities relative to chloride were not determined. Even glutamate and isethionate were permeant, although much less so than chloride. If this interpretation is valid, the delayed current is not carried by chloride alone but by a variable mix of organic as well as inorganic anions.

Table 2. Relative permeability of anions[a]

Anion	Relative permeability
Nitrate	1.5
Chloride	1
PIPES	0.5–0.8
Sulfate	0.2
Succinate	0.15

[a] At the external pH (6.8) used throughout these experiments, half of the PIPES (5 mM) is a zwitterion with a net charge of zero and half has a single net (negative) charge. Both sulfate and succinate are divalent anions at pH 6.8.

Experiments analogous to those shown in Fig. 11 were repeated with cells grown on Sr^{2+} in place of Ca^{2+}, and also incubated in the presence of 1 mM Sr^{2+} (Ca^{2+} absent). Strontium did not permit either nitrate or phosphate to flow across the membrane at positive membrane potentials (*data not shown*). We conclude that these ions, like chloride, pass through a channel activated by the entry of Ca^{2+} ions. Whether Ca^{2+} entry triggers the opening of a single kind of channel of broad specificity or a multitude of narrower ones cannot be determined from those data, but for the sake of simplicity we assume that there is a single Ca^{2+}-activated anion channel.

Osmotic Shock Elicits Action Potentials

The action potentials characterized above were elicited by the injection of depolarizing current into cells immersed in buffered $CaCl_2$. The effect could be readily produced in cells growing in either complex or defined media, in vegetative cells from five to seventeen hours old, even in sporulating cells to within a half hour of zoospore discharge and in resistant sporangia (thick-walled, dormant cells). However, numerous attempts to observe spontaneous action potentials in various media, or to elicit them by a variety of stimuli, met with little success. Aside from current injection, only two treatments were effective. Action potentials were sometimes elicited by abrupt depolarization of the plasma membrane; for instance, by replacing the medium with one containing 100 mM KCl, or with a mixture of amino acids (depolarization by amino acids is probably due to entry by symport with protons; Sanders et al., 1983). A somewhat more suggestive stimulus is osmotic shock: abrupt reduction of the external osmolarity (downshock) elicited one or more action potentials; an equivalent upshock had no effect.

A typical result is shown in Fig. 12*A*. In this experiment, the cells were first exposed to buffered

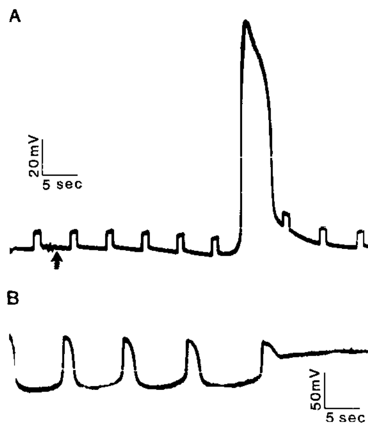

Fig. 12. Action potentials generated in response to an abrupt reduction of the extracellular osmolarity. *A*. Chloride-grown cells. Cells were grown on PYG medium supplemented with 1 mM $CaCl_2$. After the cell had been impaled, the growth medium was replaced by fresh PYG plus 1 mM $CaCl_2$ and 100 mM sucrose. The cell was allowed to adapt to this medium for 30 min (continuous perfusion; resting potential was −84 mV). At that time (arrow), the perfusion solution was replaced by PYG + 1 mM $CaCl_2$ (no sucrose). A train of small depolarizing pulses was applied continuously to monitor membrane resistance before, during and after the action potential. *B*. Chloride-free cells. Cells were grown in chloride-free DM2 and then incubated for 30 min in the same medium plus 100 mM sucrose. After the cell had been impaled (resting potential was −61 mV), the chamber was perfused with fresh chloride-free DM2 (no sucrose). This cell generated six successive action potentials, of which five are shown

0.1 M sucrose and 1 mM $CaCl_2$ for 30 min; the medium was then replaced by the usual buffered 1 mM $CaCl_2$, thereby reducing the osmotic pressure by 100 mOsm while leaving the ionic composition of the medium unchanged. This particular cell responded by firing a single action potential; others fired repeatedly, and the action potentials sometimes lasted for 25 to 30 sec. Action potentials in response to downshock were seen only in the presence of Ca^{2+} ions and were blocked by the addition of La^{3+} (50 μM). Osmotic downshock failed to elicit action potentials in cells grown on media that contained Sr^{2+} in place of Ca^{2+}. By contrast, cells grown in the absence of chloride readily produced action potentials in response to osmotic downshock; Fig. 12*B* depicts a train of such events. Evidently, action potentials elicited by osmotic downshock involve the same ion channels as those induced by current injection.

Is the Anion Channel Involved in the Regulation of Turgor?

In order to test this proposition, we examined the loss of ^{36}Cl from *B. emersonii* in response to osmotic downshock. Cells were loaded with ^{36}Cl in the presence of 0.1 M sucrose; when the medium was replaced with one devoid of sucrose, 30 to 60% of the ^{36}Cl was lost within 60 sec. However, the characteristics of ^{36}Cl efflux were quite unlike those of the action potential: external Ca^{2+} ions were not required, La^{3+} had no effect and 0.5 mM ethacrynic acid inhibited it only by half. The extent and time course of efflux were the same for cells grown on Ca^{2+} and on Sr^{2+} ions. This suggests that osmotically induced chloride efflux involves another pathway, probably one that does not translocate charges across the plasma membrane.

Only when chloride was omitted from the medium were there indications that the anion channel participants in turgor regulation. Cells grown on Ca^{2+}-PIPES medium were easily damaged by osmotic downshock, particularly when La^{3+} was added; and Sr^{2+}-PIPES medium (unlike a Sr^{2+}-chloride medium) failed to support normal growth. One possible interpretation is that such cells employ an anionic osmolite that can only exit via the calcium-controlled anion channel.

Discussion

Ion channels that open in response to Ca^{2+} have been known for over twenty years (Findlay & Hope, 1964). Calcium-activated potassium channels appear to be ubiquitous in animal cells (Meech, 1978); calcium-dependent anion channels have received less attention but they, also, are widely distributed. This paper describes the characterization of calcium and anion channels in *Blastocladiella emersonii*, a unicellular water mold that lends itself both to electrophysiological experiments and to manipulation of the conditions of growth.

A Calcium Channel and a Calcium-Dependent Anion Channel

Injection of depolarizing current into vegetative cells of *Blastocladiella emersonii* elicits a regenerative response that has the electrical characteristics of an action potential. Once they have been taken past a threshold of around −40 mV, cells abruptly depolarize to potentials ranging from +20 to +75 mV in different cells and, after a variable time (from a few hundred milliseconds up to several seconds), spontaneously return to the resting potential. We interpret these findings by analogy with the extensive work on channel-mediated ion transport in ani-

mal cells, and propose that the physical basis of the action potential consists of a calcium channel and a calcium-controlled anion channel (Fig. 13). Current injection elicits the opening of voltage-sensitive channels that allow an influx of Ca^{2+}, Sr^{2+} and Ba^{2+} ions across the plasma membrane. Calcium ions, in turn, elicit the opening of anion channels of fairly broad specificity that pass chloride, nitrate and a variety of small inorganic and organic anions. Since the anion channels open in response to an influx of Ca^{2+} but not of Sr^{2+} or Ba^{2+}, we infer that the two channels are connected by a calcium-specific receptor. Figure 13 depicts the two channels adjacent to one another, but there is no experimental evidence on this point.

The chief observations that support this hypothesis are the following. (i) The action potential requires external Ca^{2+} or Sr^{2+} ions, and is blocked by Co^{2+}, La^{3+} and by other lanthanide ions. (ii) Current flow during the action potential is kinetically biphasic; both Ca^{2+} and Sr^{2+} ions support the early phase, which we identify with the opening of voltage-sensitive divalent cation channels. (iii) The delayed current, larger and more prolonged than the initial one, is observed in the presence of Ca^{2+} ions but not of Sr^{2+} ions. (iv) The electrical characteristics of the delayed current suggest that it is carried largely, but not entirely, by chloride efflux. Other ions that can flow in response to calcium entry include nitrate, phosphate, acetate and succinate. We assume that all these anions flow through a single channel, but cannot exclude the existence of multiple, Ca^{2+}-controlled channels of narrower specificity. (v) Cells grown in the absence of chloride can still generate action potentials in response to current injection. (vi) We have found no evidence that the flux of other ions such as K^+ or H^+, contributes significantly to the action potential. Because of its broad specificity (Table 2), we speak of an anion channel rather than a chloride channel.

The factors that control the opening and closing of the two channels have yet to be explored. Activation of the calcium channels is clearly voltage dependent (Fig. 3), but we do not know what inactivates them. Ca^{2+} channels may close spontaneously during prolonged depolarization, or in response to the increase in cytosolic calcium concentration. The anion channels cannot be opened by depolarization alone (Fig. 5) and require Ca^{2+} for activation. Once Ca^{2+} has activated the anion channels, however, their opening may be influenced by the membrane potential. Inactivation of the anion channel requires hundreds of milliseconds; it could be a direct consequence of depolarization, but it is more likely due to the sequestration or ejection of the cytosolic calcium ions.

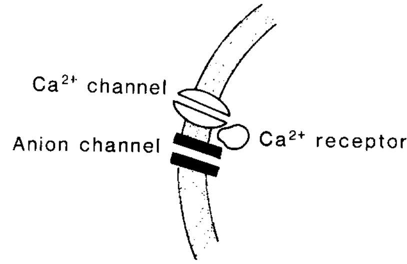

Fig. 13. Diagram of the calcium-controlled anion channel. We propose that the two channels are closely juxtaposed and are linked by a calcium-selective receptor protein

Channel Selectivity

The selectivity of the calcium channel has already been mentioned; it resembles that of calcium channels in a variety of other cells (Hagiwara & Byerly, 1981). The calcium-dependent anion channel, however, is unusual in the lack of selectivity among various anions while strongly preferring anions to cations. Because the anion current could be generated by cells that had neither internal nor external chloride, we sought other natural anions that pass through this channel. Permeant anions were found to include large anions, such as PIPES, and divalent ones such as sulfate. Such a broad range is uncommon but not without precedent. Chloride channels (Ca^{2+} independent) of muscle and brain cells are generally impermeant to divalent anions (Edwards, 1982), and the calcium-dependent chloride channels of neurons (Owen et al., 1984) and of oocytes (Miledi, 1982), exclude sulfate and organic anions. Cortical neurons, however, admit large anions (Kelly et al., 1969) and Schwann cells of the rat possess high-conductance anion channels that are permeable to both sulfate and methyl sulfate (Gray et al., 1984). The Ca^{2+}-dependent anion channel described here passes not only chloride, PIPES and sulfate, but also phosphate, acetate, nitrate and succinate.

Physiological Function

The function of the calcium-controlled anion channel is not known; speculation must take account of the following observations that complement the biophysical characterization of the ion fluxes.

(1) Action potentials have been elicited in cells as young as six hours (diameter 14 μm), throughout the vegetative phase and the sporulation cycle, and even in resistant sporangia. They have been observed in both complex and defined media of vari-

ous compositions, and must be taken to be a general capacity of the organism.

(2) We have occasionally observed spontaneous action potentials, but they are rare. They do not occur regularly at any stage of growth or of sporulation under our conditions, nor have we been able to elicit action potentials by shifting the cells from one medium to another, with two exceptions: treatments that cause rapid depolarization of the plasma membrane, and a sudden reduction of the extracellular osmotic pressure. The latter observation hints at an involvement of the calcium and anion channels in the control of cell turgor, but may be deceptive: an osmotic shock, no less than a jolt of depolarizing current, may artificially force open a calcium channel that normally responds to entirely different stimuli.

(3) *Blastocladiella* grows perfectly well under conditions such that the action potential cannot be elicited, even by current injection. Only 10 μM Ca^{2+} are required for normal growth and sporulation, but the action potential requires the presence of 30 μM Ca^{2+}, at least. By the same token, Sr^{2+} ions support normal growth and sporulation, yet do not permit opening of the anion channel. These observations suggest that neither the action potential nor the anion channel has an obligatory role in growth or sporulation, at least under laboratory conditions. Indeed, the action potential as described here is probably an artifact that rarely (if ever) occurs naturally.

(4) On the other hand, *Blastocladiella* does require a minimal level of external Ca^{2+} ions for both growth and sporulation. Moreover, La^{3+} and a series of other lanthanide ions block growth and sporulation at the same levels that block the action potential. [In PYG plus 1 mM Ca^{2+}, these levels are (in mM): La^{3+} 0.3, Tb^{3+} 0.5, Y^{3+} 0.5, Yb^{3+} 0.5, Ga^{3+} 0.5, and Ce^{3+} 2. In media devoid of phosphate, the effective lanthanide concentrations are much lower.] It is possible that these effects involve a different set of calcium channels or binding sites, but we prefer the parsimonius view that the calcium channel described here performs some function essential to growth and sporulation. It is widely believed that fungi and plants grow by the calcium-controlled exocytosis of precursor vesicles (Nuccitelli & Jaffe, 1976; Gooday, 1983); this may be the function of the calcium channels.

The calcium-controlled anion channel of *Blastocladiella* resembles those found in the algae *Chara, Nitella, Pelvetia* and *Acetabularia,* which have been implicated in the control of turgor. There is substantial evidence that in these organisms excessive turgor opens the calcium channels, and the subsequent efflux of chloride relieves the pressure (Findlay & Hope, 1964; Nuccitelli & Jaffe, 1976; Zimmermann & Beckers, 1978; Wendler et al., 1983). Calcium-controlled chloride fluxes have also been implicated in volume regulation in some animal cells (Grinstein et al., 1982; Hoffman et al., 1984). Despite these precedents, and our own observation that osmotic downshock elicits action potentials in *Blastocladiella* (Fig. 12), we doubt that this is the primary function of our calcium-dependent anion channel. The reasons are as follows. (1) Chloride flux can relieve turgor pressure only if accompanied by a cation; yet we saw no sign of K^+ efflux during an action potential. (2) Osmotic downshock induced chloride efflux from *Blastocladiella* even when the calcium-controlled anion channel was blocked (for example, in strontium-grown cells or in the presence of ethacrynic acid). This suggests that the cells cope with excessive turgor by means of another, electroneutral pathway of chloride flux. Only under extreme conditions does the calcium-controlled anion channel appear to be required for turgor regulation; for example, in cells grown in media that contain PIPES in place of chloride.

We suspect that the calcium and anion channels form an integrated complex that functions locally in the control of vesicle exocytosis. There is some evidence that fusion of exocytotic vesicles with the plasma membrane requires an osmotic gradient as well as calcium ions (Akabas et al., 1984): could the anion channel help regulate the local turgor pressure? Alternatively, opening of the anion channel could regulate the flux of calcium ions in more than one way. Anion efflux may depolarize the membrane in the immediate vicinity of the Ca^{2+} channel, and this may limit the influx of calcium ions by reducing the electrochemical driving force. On the other hand, the opening of the anion channel may activate neighboring Ca^{2+} channels and thus allow the depolarization to spread beyond its point of origin. All these notions are too tenuous to be developed in detail; they are mentioned here in order to indicate that the calcium action potential described above, albeit an artifact, may reflect a more localized phenomenon that plays a significant role in the growth of walled cells.

This paper is dedicated to the memory of Edward C. Cantino, who domesticated *Blastocladiella emersonii* and devoted his life to its study. We thank Dr. A.R. Martin for designing the voltage clamp, Mark Lupa for building it and participating in the early experiments, Diane Nicholl for technical assistance and Ruth Harold for drawing Fig. 1. Supported in part by grants No. AI-03568 from the National Institutes of Health and PCM 8009439 from the National Science Foundation.

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