Transcellular Ion Currents during Sporangium Development in the Water Mould *Achlya bisexualis*

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Changes in the pattern of electric currents that accompany the transformation of growing hyphae of *Achlya bisexualis* into sporangia have been examined. When hyphae were transferred to a non-nutrient buffer, they continued to extend for several hours and then gave rise to sporangia. Throughout this process, current (positive charge) flowed into the apical region that corresponds approximately to the future sporangium. The current ceased after the crosswall appeared. The sporangium then remained electrically quiescent, except for a brief intense burst of outward current at the 'homogeneous' stage of spore cleavage. The inward current during sporangium formation largely represents an influx of protons. Addition of nitrate abolished the flow of electric current with little effect on sporulation. The late burst of outward current is most probably an artefact, generated by the discharge of salts from the sporangial vacuole. The transcellular electric current apparently plays no role in sporangium formation or in spore cleavage. Calcium ions, however, are required and may traverse the plasma membrane.

INTRODUCTION

Eukaryotic cells and organisms commonly drive ionic currents through themselves, due to the non-uniform distribution of ion-transport systems in the plasma membrane. The biological significance of these currents is not fully understood, but they are thought to play important roles in cellular physiology, especially in the spatial localization of growth and development (Jaffe & Nuccitelli, 1977; Jaffe, 1981; Nuccitelli, 1982, 1986). This report is part of a systematic study of the genesis and functions of transcellular ion currents in the oomycetous water mould *Achlya bisexualis*.

Ionic currents associated with hyphal growth and branching have been extensively studied (Kropf *et al.*, 1983, 1984; Gow *et al.*, 1984; Kropf, 1986; Harold *et al.*, 1987; Gow & Gooday, 1987; Schreurs & Harold, 1988). In growing hyphae current (positive charges) enters the apical region and exits distally. The current arises by the spatial segregation of proton pumps from amino acid porters. Amino acid porters are particularly abundant in the apical zone, while the H⁺-ATPase may be excluded from the apex. This arrangement results in a net influx of protons into the hyphal anterior; acidification may play a role in the physiology of extension.

Transfer of growing hyphae into a solution of salts devoid of carbon and nitrogen directs them into a developmental pathway that culminates in sporulation (Gay & Greenwood, 1966; Heath, 1976; Armbruster, 1982*a, b*). The hyphae continue to extend for a time but then elongation ceases and the tip rounds up. Cytoplasm moves forward into the apical region, which becomes dense and turgid. Formation of a crosswall delimits the developing sporangium, and later a papilla forms at the tip. Concurrently, the cytoplasm cleaves into uninucleate spores that are ultimately discharged through the papilla. Armbruster & Weisenseel (1983), working with

Achlya debaryana, reported that development of the sporangium is attended by continuous electric current, flowing into the hypha except for a burst of outward current at the 'homogeneous' stage of spore cleavage.

In this paper we describe experiments to characterize the electric currents associated with sporangium development, and to gain some insight into their physiological significance.

METHODS

Organisms and media. Procedures for growing and maintaining *Achlya bisexualis* T5 were described by Kropf *et al.* (1984); zoospores for use as inocula were produced by the method of Gow *et al.* (1984). For the present experiments hyphae were grown on PYG medium (Bacto-peptone, 1.25 g l⁻¹; yeast extract, 1.25 g l⁻¹; glucose, 3 g l⁻¹; Bacto-agar, 20 g l⁻¹). A drop of spore suspension was placed in the centre of a thin PYG agar plate (11 ml in a 100 mm Petri dish) and the plate was incubated at 23 °C for about 48 h, producing a radial colony.

To induce sporulation, small plugs were cut from the edge of the colony and placed in buffered CaCl₂ (Griffin, 1966). A solution containing 0.25 mM-CaCl₂, 0.25 mM-KCl and 1 mM-Tris/PIPES, pH 6.5, gave the best results. One hour after this transfer the sporulation buffer was replaced with fresh sporulation buffer, and incubation was continued at 23 °C or at room temperature. Hyphae continued to extend at 1–3 µm min⁻¹ for several hours and then rounded up. The crosswall usually became visible 30–60 min after extension ceased, the papilla appeared after another hour, and zoospores were released about 30 min after papilla formation.

Measuring the electric current. The procedure used here (Schreurs & Harold, 1988) is a modification of that described by Kropf *et al.* (1984). Circular agar blocks cut from the edge of a colony were glued to coverslips and incubated in sporulation solution as described above, until a few hyphae had grown off the edge of the plug. The coverslip was then attached to the bottom of a recording chamber, and currents were monitored by use of a vibrating probe (Jaffe & Nuccitelli, 1974). Media were exchanged with the aid of two syringes coupled together such that filling one empties the other (Kropf *et al.*, 1984).

Mapping the extracellular pH. The procedures were modified from those of Gow *et al.* (1984). Hyphae were grown on Nuclepore filters (8 µm pore size); a small piece of filter bearing a few isolated hyphae was then transferred to a recording chamber and held in position with a plastic chromatography clip. Electrodes were pulled from Boralex micropipettes (100 µl; Rochester Scientific Co.), to a tip-size of 10 µm. Baked and silanized electrodes were filled with a small volume of 'proton cocktail' (Fluka) and then backfilled with a solution of 40 mM-KH₂PO₄, 23 mM-NaOH and 15 mM-NaCl, pH 7 (Ammann *et al.*, 1981); the output in mV was measured with a high-impedance electrometer and converted into pH units.

Other methods. Tip extension was monitored under the microscope in the recording chambers mentioned above. To measure ammonia production, mycelium was grown on Nuclepore filters (pore size 8 µm) floating on PYG medium as described by Kropf & Harold (1982). The filters were then rinsed, transferred to sporulation buffer, and the NH₃ content of the buffer was assayed after 5 h by use of a specific electrode (Microelectrodes, Inc., Londonderry, NH, USA).

Reagents. The calcium-channel blockers Nifedipine and BAY K8644, manufactured by Bayer, were donated by Dr Alexander Scriabine of Miles Laboratories, Inc., New Haven, CT, USA. Verapamil and unspecified reagents were purchased from Sigma.

RESULTS

Transcellular currents and the course of sporulation

Fig. 1 displays consecutive maps of the electric current generated by a sporulating hypha. This particular hypha extended slowly for 4 h after transfer to sporulation buffer, driving current into a broad apical zone. Inward current continued after extension stopped, but gradually declined and ceased upon completion of the crosswall that delimits the sporangium (5–6 h). No subsequent electrical activity was observed in this experiment.

Inward current was a characteristic feature of sporangium development, but varied greatly in intensity: measured values ranged from 0.01 to 0.31 µA cm⁻² (mean $\pm$ SD 0.14 $\pm$ 0.08 µA cm⁻²; $n = 97$); when these values are extrapolated to the cell surface by the formula of Kropf *et al.* (1984), one estimates a density near 1 µA cm⁻². Eight of the sporangia mapped in this manner showed a burst of outward current late in sporulation (see below).

Fig. 2 shows the time-course of electric current registered by a probe positioned continuously in front of the sporangial tip. Inward current rose steadily to a maximum at about 4 h; it diminished after the crosswall became visible and ultimately ceased. The sporangium then remained electrically quiescent, except for the brief, biphasic burst of intense outward current

Fig. 1. Electric currents during sporulation. Hyphae were transferred to sporulation buffer at 0 h and successive current maps of a single hypha were taken at intervals thereafter. The ordinate represents the magnitude of current normal to the hyphal axis. In this particular hypha the crosswall formed 320 μm behind the tip (arrow). The sketches are not to scale.

corresponding to the 'homogeneous' stage of spore cleavage. This burst was seen in eight out of nine sporangia mapped continuously. These observations are in general agreement with those of Armbruster & Weisenseel (1983), except that the electric current was steady rather than spiky.

Outward current during the 'homogeneous' stage represents potassium efflux

Money & Brownlee (1987) have reported that the 'homogeneous' stage is marked by biphasic efflux of K^+ from the sporangium, which they detected by use of a K^+ -selective microelectrode.

Fig. 2. Time-course of the electric current during sporulation. Hyphae were transferred to sporulation buffer at 0 h. Current flow into a single hypha was monitored at intervals thereafter, with the vibrating electrode positioned just in front of the tip. Intervals when the electrode was in the reference position are marked R. Shown here is an idealized record, obtained by plotting readings in the reference position as zero current. Note the cessation of inward current after crosswall formation, and the biphasic burst of outward current at 6.1 h.

Fig. 3. Pulses of KCl ejected from a microelectrode are registered as outward current by the vibrating probe. For details, see text.

K⁺ efflux probably signals the emptying of the central vacuole, as the developing zoospores acquire individual plasma membranes (Gay & Greenwood, 1966; Armbruster, 1982*b*). Efflux of salts may also account for the apparent burst of outward electric current at this stage (Fig. 2): it gives rise to a transient increase in the conductivity of the medium adjacent to the sporangium, which the vibrating probe registers as an outward current.

Fig. 3 shows an experiment in which successive pulses of 1 mM-KCl were ejected from a micropipette by pressure pulses generated with a picospritzer (General Valve Corporation,

Fig. 4. The electric current is not required for sporulation. Hyphae were transferred to sporulation buffer at 0 h, and a single one was monitored with the vibrating electrode positioned in front of the tip. At 5 h, as soon as the tip had rounded up, the sporulation buffer was exchanged for fresh buffer supplemented with 3 mM-KNO₃. Spores were discharged at 6.5 h. Intervals when the probe was in reference position are designated R; the record is idealized as in Fig. 2.

Fairfield, NJ, USA). The nearby vibrating probe read each pulse as a burst of outward current. By the use of micropipettes filled with [¹⁴C]arginine, we estimated that each pulse in Fig. 3 reports the ejection of 2 nl from the pipette, or about 2 picequivalents of KCl. The pulse generated by a typical sporangium corresponds to the discharge of a somewhat larger amount of KCl. Sporangia are commonly about 200 μm long and 30 μm in diameter (volume 140 pl). Assuming that the vacuole occupies a third of this volume and is filled with 100 mM-KCl, its discharge would yield 5 picequivalents of KCl. The correspondence of these magnitudes reinforces the belief that the burst of 'outward current' produced during the 'homogeneous' stage is an artefact resulting from salt discharge.

Proton flow during sporangiogenesis

Formation of the sporangium is attended by an inward current that continues until the crosswall has been completed (Figs 1 and 2). To identify the ions that carry the current we investigated the effects of modifying the ionic composition of the medium. Neither sporulation nor the electric current were altered by omission of the buffer or by replacement of K⁺ with Tris (data not shown). Substitution of PIPES for Cl⁻ did alter the current pattern: hyphae extended for several hours while driving an outward current, which gradually gave way to the normal pattern of inward current (data not shown). Sporulation was somewhat delayed but otherwise normal. Current patterns and sporulation were unaffected by pH over the range from 6.5 to 8.5, but more alkaline conditions were inhibitory. We infer that neither K⁺ nor Cl⁻ is required to carry the inward current, which probably represents chiefly an influx of protons.

Is the transcellular flow of electric current required for sporulation? Quite by chance we noted that addition of certain anions, NO₃⁻ in particular, often abolished the flow of charge. Fig. 4 shows an experiment in which hyphae were allowed to develop into sporangia in standard sporulation buffer; 3 mM-KNO₃ was added as soon as extension ceased. The transcellular current quickly diminished and then ceased, but sporulation was completed on time. The effect

Fig. 5. Extracellular pH profile during sporulation. At 0 h hyphae were transferred to sporulation solution modified to reduce the buffer capacity (0.25 mM- CaCl_2 , 0.25 mM-KCl, 25 μM -PIPES, pH 6.50). Extracellular pH was mapped at intervals as described in Methods. The ordinate represents the difference between the pH adjacent to the hypha and that of the bulk medium. This hypha discharged spores at 6.75 h.

of NO_3^- was not fully reproducible (out of 11 hyphae, NO_3^- abolished the current in 7), nor do we understand its mechanism; it is nevertheless clear that a transcellular electric current is not required for development of the sporangium.

Alkalinization and ammonia formation

Hyphae growing in nutrient medium generate a longitudinal pH gradient, such that the medium adjacent to the apical region is slightly alkaline while that surrounding the trunk is acidic. Apical alkalinity was formerly attributed to proton influx (Kropf *et al.*, 1984; Gow *et al.*, 1984), but we now suspect that it reports chiefly the production of ammonia (Schreurs & Harold, 1988).

Hyphae transferred to sporulation buffer generated a distinctive pH profile (Fig. 5). The medium surrounding the future sporangium became markedly alkaline, but little acid was produced along the trunk. The pH gradient subsided and eventually vanished after completion of the crosswall. The alkaline zone is too marked to reflect the weak proton current, but could well be attributed to NH_3 released from protein catabolism. Indeed, hyphae generate NH_3 when placed in non-nutrient medium: a Nuclepore filter bearing hyphae grown from 2×10^4 spores, immersed in sporulation buffer, produced 8.3 nmol NH_3 in the course of 6 h. In the absence of ammonia-selective microelectrodes we cannot localize the site of NH_3 formation, or even be sure that it is formed by hyphae engaged in sporulation, but apical generation of NH_3 is now the most plausible explanation for apical alkalinity.

Sporulation requires calcium ions

It is known that micromolar concentrations of Ca^{2+} are required for sporulation (Griffin, 1966). When hyphae were placed in sporulation buffer without any added Ca^{2+} (1 mM- K^+ -PIPES, 0.25 mM-KCl; contaminating Ca^{2+} , 1–5 μM), about half of them formed sporangia and ultimately produced spores; inward current was essentially normal, $0.4 \mu\text{A cm}^{-2}$. Reduction of

the free Ca^{2+} concentration below $0.1 \mu\text{M}$ by the addition of 0.1 mM -EGTA blocked sporulation and caused some of the hyphae to lyse at the tips; inward current increased by as much as tenfold, to $6.3 \mu\text{A cm}^{-2}$. The findings suggest that Ca^{2+} ions do not make a large contribution to the inward electric current, but their presence is required to maintain normal ion permeability.

La^{3+} ions are known to block calcium channels. When $20 \mu\text{M}$ - LaCl_3 was added 3.5 h after transfer of the hyphae to sporulation buffer (0.25 mM - CaCl_2 present), sporulation was blocked: no crosswall, papilla or spores were seen. The transcellular current was enhanced somewhat (from 0.1 to $0.2 \mu\text{A cm}^{-2}$), suggesting again that Ca^{2+} influx makes but a minor contribution to the current. A series of experiments of this kind (not illustrated) showed that addition of $20 \mu\text{M}$ - LaCl_3 to extending hyphae stopped their elongation within 1–10 min, and blocked all subsequent stages of sporulation. When added after the tips had rounded up, La^{3+} again blocked the later stages; and when La^{3+} was added after the crosswall had become visible, the discharge papilla usually appeared but no spores were produced. Gd^{3+} ions at $50 \mu\text{M}$ had similar effects; crosswall and papilla formation were greatly delayed, and spores did not form. The organic calcium-channel blockers tested (all at 0.1 mM) appeared to be generally toxic, eliciting lysis and vacuolation rather than a clear blockade of sporulation.

DISCUSSION

The most general conclusion to emerge from our earlier studies on transcellular ion currents in *Achlya* is that, so long as a hypha extends, protons flow into its apical zone. Proton influx is detectable by virtue of the attendant inward electric current. Under special circumstances the net flow of charge may be masked by other ion movements, but the underlying proton influx may persist even then. The present experiments illustrate this phenomenon yet again. Hyphae transferred to CaCl_2 solution devoid of nutrients continue to extend for several hours and then stop; the apical region then rounds up and progressively develops into a sporangium. During this entire time an electric current, apparently carried largely by protons, flows into a long apical zone that corresponds approximately to the future sporangium. Proton influx diminishes when the crosswall becomes visible, and then ceases (Figs 1 and 2).

Generation of a transcellular proton current in nutrient media has been attributed to the interplay of two transport processes: amino acid/ H^+ symport, preferentially localized in the hyphal anterior, and proton extrusion by the H^+ -ATPase, which is thought to be more abundant along the trunk (Schreurs & Harold, 1988). Sporulation buffer lacks organic nutrients and its major ionic constituents (K^+ , Ca^{2+} , Cl^-) appear to make little contribution to the inward current. If, indeed, this current represents the net influx of protons, it may be due to the 'leakage' of protons into an apical zone relatively deficient in proton-extruding ATPase. This hypothesis must now be tested experimentally.

Is the electric current, or the influx of protons, *required* for sporangium development? Addition of NO_3^- often abolished the transcellular electric current without ill effect on sporulation, suggesting that this developmental transformation does not depend on the electric current (Fig. 4). Incidentally, in sporulation buffer devoid of Cl^- , hyphae extend for hours while driving outward, rather than inward, current through the apical region. This finding is reminiscent of the current reversal that is sometimes seen during branching (Kropf *et al.*, 1983), and reinforces our view that hyphal extension does not require inward current either (Harold *et al.*, 1987; Schreurs & Harold, 1988). It is much more difficult to determine whether influx of protons persists under these conditions, and whether it is required, as we can only track the flow of protons through the attendant flow of charge. The alkaline zone surrounding the developing sporangium (Fig. 5), which might earlier have been attributed to proton uptake (as was done in the case of extending hyphae; Gow *et al.*, 1984), is more probably due to NH_3 production. Extension and sporulation are both inhibited at pH 9, but that is a weak argument for dependence on proton influx. We can therefore conclude only that the transcellular electric current plays no obligatory role in sporulation. Proton influx may persist even in the presence of NO_3^- , and may be physiologically significant, but its study must await the development of novel instruments that track the movement of particular ions.

Another electrical feature of sporulation is the burst of outward current that marks the 'homogeneous' stage of spore cleavage (Armbruster & Weisenseel, 1983). An analogous current pulse was previously noted in *Blastocladiella* by Stump *et al.* (1980). The evidence now indicates that this 'current' is an artefact, due to the discharge of salts from the central vacuole at the time the spores acquire individual plasma membranes (Money & Brownlee, 1987). The apparent 'current' is probably of interest chiefly as a marker of ion efflux, and as a warning to users of the vibrating probe.

Of all the phenomena studied here, the only one that is clearly of developmental significance is the requirement for Ca^{2+} . In *Achlya*, as in *Blastocladiella*, micromolar concentrations, at least, must be present for the sporangium to develop and for spores to form (Griffin, 1966; Lovett, 1975; Caldwell *et al.*, 1986; Money, 1986). La^{3+} ions inhibit the process, and there are indications that Ca^{2+} ions are required throughout the course of sporangium development and spore maturation. Unfortunately, just what this Ca^{2+} requirement represents remains entirely obscure. Ca^{2+} ions must apparently be present to maintain the normal ionic permeability of the plasma membrane, particularly its K^{+} conductance (Slayman, 1965, and personal communication; Caldwell *et al.*, 1986). The massive inward current seen in the presence of EGTA probably represents an abnormal ion flux unmasked by Ca^{2+} depletion. This relatively general function may be satisfied by extracellular Ca^{2+} , since it is unaffected by the presence of low levels of La^{3+} . La^{3+} ions do, however, inhibit extension as well as the ensuing stages of sporulation. It is reasonable to speculate that La^{3+} ions inhibit some critical transport step, possibly blocking Ca^{2+} channels such as those detected in *Blastocladiella* (Caldwell *et al.*, 1986). Calmodulin, which is found particularly in the apical region of hyphae and of developing sporangia (Suryanarayana & Des S. Thomas, 1986), may function as the receptor for Ca^{2+} ions that enter. Just what roles Ca^{2+} and calmodulin actually play remains to be discovered.

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