The Transhyphal Electrical Current of *Neurospora crassa* is Carried Principally by Protons

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During apical extension an ion current of about 0.2 μA cm⁻² flows into the hyphal tips of *Neurospora crassa* and out from the distal regions of the hyphae. This current is carried principally by protons and requires phosphate and glucose in the growth medium. Exogenous calcium ions were required for tip extension but not for the current. A permeable pH sensitive dye, bromocresol green, provided evidence that the cytoplasm at sites of proton current entry was made acidic in comparison with the subapical cytoplasm. In a few cases non-growing hyphae were found with a normal current and growing hyphae were found with outward current at the apex. These current patterns, although rare, indicate there is no tight correlation between the vectorial flow of electrical current and hyphal extension in this organism.

**INTRODUCTION**

Ion transport proteins in the plasmalemma of eukaryotic cells are commonly organized so that there is a net flow of electrical charge through the cytoplasm and in the extracellular space. This electrical circulation can be detected and mapped extracellularly using a vibrating probe (Jaffe & Nuccitelli, 1974). In polarized cells and tissues where growth occurs by apical extension such as in fungal hyphae, pollen tubes, algal rhizoids, plant roots, root hairs, and nerve axons and dendrites, positive electrical current invariably enters the growing apex (Gow *et al.*, 1984; Weisenseel *et al.*, 1975, 1979; Robinson & Jaffe, 1975; Freeman *et al.*, 1985). This has led to the hypothesis that ionic currents and associated electrical fields may be essential components of the mechanism or mechanisms which bring about polarized growth. Although ionic currents have now been mapped in a diversity of systems (Nuccitelli, 1983) there is much less information regarding the ionic composition of these currents. In the *Pelvetia* egg Ca²⁺ influx and Cl⁻ efflux carry inward current (Robinson & Jaffe, 1975; Nuccitelli & Jaffe, 1976) and in lily pollen germ tubes K⁺ carries inward current and H⁺ outward current (Weisenseel & Jaffe, 1976). The current of barley roots would seem to be carried mainly by protons (Weisenseel *et al.*, 1979). Although transhyphal currents have been recorded in representative species of fungi from all the major taxonomic ranks (Armbruster & Weisenseel, 1983; Kropf *et al.*, 1983; Gow, 1984; Horwitz *et al.*, 1984) only that of the water mould *Achlya bisexualis* has been examined in detail. Here, current entry is probably due to proton/amino acid symport and outward current proton efflux generated by a proton pumping ATPase located in the distal plasmalemma (Kropf *et al.*, 1984; Gow *et al.*, 1984; Kropf, 1986). The current in *A. bisexualis* can therefore be regarded as a spatially extended chemiosmotic system with proton pumps at the rear of hyphae and proton leaks at the apex (Harold *et al.*, 1985a, b).

*Neurospora crassa* has also been shown to generate a transhyphal electrical current (Gow, 1984). This finding consolidates an early electrophysical study which showed that there was a gradient membrane potential between the apex and distal regions of hyphae of this organism, so that the apex was relatively depolarized (Slayman & Slayman, 1962). This paper describes the
preliminary characterization of the ion current of *N. crassa*, and concludes that this current is carried mainly by protons.

**METHODS**

*Organism and media.* Neurospora crassa RL21A was obtained from C. L. Slayman (University of Yale, Conn., USA) and was maintained and grown in Vogel's minimal medium (Vogel, 1956) modified so that all salts were at one-tenth the normal concentration but with glucose and biotin at the normal concentration. The resistivity of this medium was typically 800–850 Ω cm. The reduced salts concentration increased the medium resistivity, thereby improving the signal to noise ratio of measurements made with the vibrating probe. Some measurements were made with the fungus growing in media containing 20 g malt extract 1⁻¹ and 10 mM-potassium phosphate buffer, pH 6.0.

*Measurements of transhyphal currents.* Hyphae were grown from an agar block which was anchored with a non-toxic quick-setting gum to the bottom of a specimen chamber with glass base as described by Gow (1984). Current measurements were made with a one-dimensional vibrating probe purchased from the Vibrating Probe Co., (Davis, Calif., USA) using a Princeton Applied Research 5101 lock-in amplifier. The vibration frequency was typically between 200 and 400 Hz and the vibration amplitude was fixed at 30 μm. The instrument and method for calculation of current densities is described by Jaffe & Nuccitelli (1974). Quadrature measurements were made at intervals by repeating a measurement with the lock-in amplifier 90° out of phase. In experiments where the bathing medium was exchanged (for example in ion substitution experiments) at least 100 ml of medium was allowed to pass through the growth chamber. Control experiments showed that this procedure ensured at least a $10^5$ dilution of the original medium. Specimens were examined during measurements using an Olympus inverted CK microscope at a final magnification of 100 X. The condenser diaphragm was kept open to provide minimal depth of field to enable the probe to be accurately positioned at the sides of the hyphae. Extension rates of hyphae were determined using an eyepiece micrometer and assessing the tip position at regular intervals.

*Staining of cells with pH indicator dyes.* Germinating conidia and hyphae of *N. crassa* were stained with the pH indicator dye bromocresol green (BDH) as described by Turian (1979, 1983). In other experiments bromocresol purple, methyl red, neutral red, phenyl red (all from BDH), alizarin yellow, acidine orange (Sigma) and 6,7-dihydroxy-4-methylcoumarin (Aldrich) were used. Bromocresol green was used at a 1% (w/v) concentration made up in Vogel’s minimal medium. This was found to be the maximum dilution which still produced efficient staining. A drop of culture containing germinating spores or young germings was added to an equal volume of stain and left for 10 min before examining using brightfield optics. In some experiments dinitrophenol (Sigma) was added to the culture medium and staining solution at a final concentration of 0.5 mM. The intracellular pH of cells which exhibited differential staining at the tip and distal regions was assessed only in specimens which had tapering germ tube apices and were not extensively vacuolated. Dead cells killed by boiling for 3 min or treatment with 35% (v/v) ethanol or 2.5% (w/v) formaldehyde were also treated with bromocresol green as above. Other specimens were stained with 1% (w/v) lactophenol blue or Giemsa (BDH).

**RESULTS**

*Transhyphal current profile.*

During hyphal tip extension, an electrical current of around 0.2 μA cm⁻² flowed into the apical region, while positive current left the distal region (Fig. 1). The zone of inward current typically entered for the first 200–300 μm of hypha but could extend distally for as far as could be measured (up to 500 μm in one instance). In those hyphae in which both inward and outward current could be measured there was no second zone of inward current behind the outward current. In general, only growing hyphae drove a circulating current but in two examples (out of fifty) non-growing hyphae were found with inwardly directed currents at the apex. In these cases, hyphal extension ultimately resumed. Rarely (three examples from fifty), but of particular importance, outward currents were recorded at extending apices (e.g. Fig. 2). Also, on one occasion, a hypha was mapped with inward current at one side and outward from the other (data not shown). This hypha extended normally without bending. In this organism, therefore, tip extension was only normally, not invariably, associated with a symmetrical inward current profile at this site.

*Ionic nature of the circulating current.*

A series of experiments was done in which the current density flowing into hyphal tips was measured before and after the medium was exchanged for one in which an individual
Fig. 1. (a) Profile of electrical current along a hypha of *N. crassa* of extension rate 9 μm min⁻¹. (b) Tracing of a section of the chart recording from this experiment showing five electrical field measurements used to calculate the current densities of the five points indicated in (a) by the arrows, and illustrating a typical working signal to noise ratio. The reference position (ref) is indicated and upward deflections correspond to outward current.

Table 1. Effect of the removal of exogenous ions and nutrients on the current density flowing into hyphal tips of *N. crassa*

Current densities are expressed as means of three to five measurements ± se. Regression analysis of the slopes of hyphal tip position against time produced correlation coefficients of 0-990 or better.

<table>
<thead>
<tr>
<th>Ion or nutrient removed</th>
<th>Substituting ion or compound</th>
<th>Maximum inward current density before and after substitution experiment (μA cm⁻²)</th>
<th>Current density after substitution experiments expressed as a percentage of the initial current density</th>
<th>Hyphal extension rate before and after substitution experiment (μm min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K⁺</td>
<td>Na⁺</td>
<td>0.423 ± 0.044 0.540 ± 0.200</td>
<td>120</td>
<td>14 10</td>
</tr>
<tr>
<td>Na⁺</td>
<td>K⁺</td>
<td>0.096 ± 0.007 0.096 ± 0.006</td>
<td>100</td>
<td>5 9</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>SO₄²⁻</td>
<td>0.150 ± 0.016 0.194 ± 0.003</td>
<td>129</td>
<td>14 13</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>Cl⁻</td>
<td>0.220 ± 0.001 0.094 ± 0.013</td>
<td>43</td>
<td>12 7</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>Ca²⁺</td>
<td>0.243 ± 0.001 0.153 ± 0.100</td>
<td>63</td>
<td>3 5</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>Mg²⁺†</td>
<td>0.230 ± 0.009 0.432 ± 0.143</td>
<td>187</td>
<td>12 0</td>
</tr>
<tr>
<td>H⁺*</td>
<td>Sorbitol</td>
<td>0.147 ± 0.014 0.003 ± 0.001</td>
<td>2</td>
<td>10 0</td>
</tr>
<tr>
<td>Glucose</td>
<td></td>
<td>0.183 ± 0.029 (0.080 ± 0.013)</td>
<td>0</td>
<td>8 6</td>
</tr>
</tbody>
</table>

* The pH of the medium was increased from 6.0 to 8.5 using KOH.
† EGTA (1 mM) was also added to further reduce the free Ca²⁺ concentration.
‡ Outward current.

component was omitted. (Table 1, Fig. 2). The removal of one ion was compensated for by the addition of an equivalent amount of a related ion. Sorbitol was used to substitute for glucose. The rationale for these experiments was that the current should only be affected by ions or solutes which flow through current-carrying membrane proteins. Of the major anions and cations present in the growth medium, only phosphate significantly reduced both current and extension rate. Removal of Ca²⁺ stopped extension without affecting current suggesting that this ion may be essential for tip growth but is not a major component of the circulating current. Increasing the pH of the medium (equivalent to reducing the free proton concentration) dramatically reduced the inward current density (Table 1, Fig. 2) indicating that protons carry most of the current. The removal of glucose from the medium also reduced the size of the current but without greatly affecting hyphal extension. Glucose may therefore be required for proton influx or alternatively current may require constitutively an available energy source. In summary, the transhyphal electrical current of *N. crassa* growing in Vogel's medium appeared to be carried principally by protons, and required phosphate, and the presence of extracellular glucose.
Intracellular pH gradients measured using indicator dyes

Since protons flow into the hyphal tip of *N. crassa* the intracellular pH in this region may be expected to be locally acidic. We used membrane permeable indicator dyes and pH sensitive fluorescent stains in order to test this hypothesis. Of the dyes (alizarin yellow, bromocresol green, bromocresol purple, methyl red, neutral red and phenyl red) and fluorescent stains (acridine orange, carboxy fluorescein diacetate and 6,7-dihydroxy-4-methylcoumarin) that were tested only bromocresol green produced differences in colouration indicative of local differences in pH. This dye did not permeate cells readily at low concentrations and concentrations which did permeate (>1%, v/v) also inhibited the extension of hyphae growing on agar plates or in liquid media. Interpretation of the result must, therefore, be tempered by the knowledge that this dye was toxic to the cells at the concentrations used.

The tips of the germ tubes of germinating conidia which had been stained with bromocresol green had a yellow colouration indicating a cytoplasmic pH of 3.5-4.0 while the conidial body was green or green/blue (pH 4.5-5.5) (Fig. 3b). In most specimens the difference in pH between the apex and distal hyphae appeared to be in the order of 1 to 1.5 pH units. Bromocresol green stained mature hyphae less readily, and in some cases not at all. Consequently internal pH gradients in mature hyphae were less convincingly demonstrable. An example of a hypha with an apex which was relatively acidic (yellow) compared to the distal hypha (green) is shown in Fig. 3. Since the diameter of germ tubes (5-7 μm) tended to be less than that of conidia (8-12 μm) we investigated whether the different colouration of these two regions was due to differences in the depth of stainable cytoplasm at these locations. Stains which did not change colour over the working pH range such as lactophenol blue and Giemsa did not show any significant intensity of staining at the apex and conidial body. Also specimens which had been killed by boiling for 3 min, or by exposure to 35% (v/v) ethanol or 2.5% (w/v) formaldehyde, showed no internal pH gradient when stained with bromocresol green. The proton uncoupler dinitrophenol caused the internal pH to equilibrate with the external pH and produced specimens which again had no detectable internal pH gradient (Fig. 3c). The staining pattern was therefore consistent with the hypothesis that proton influx at the hyphal apex brings about a localized acidification of the cytoplasm at this location and supports the conclusion that protons carry most of the circulating current in this organism.
DISCUSSION

The transhyphal electrical current that flows into hyphal tips appears to be carried mainly by protons. Phosphate ions appear to be important for current and hyphal extension since the removal of phosphate reduced both by 40%. If phosphate carried inward current this anion would have to be undergoing efflux at the tip to explain positive current entry. Alternatively, phosphate may be essential for growth and current generation without actually contributing directly to the electrical flux. Inward current in the water-mould A. bisexualis normally requires the provision of amino acids in the growth medium and it has been proposed that current enters by amino acid/proton symport (Kropf et al., 1984; Gow et al., 1984; Kropf, 1986). By analogy, the inward current of N. crassa requires glucose and so might operate by glucose/proton symport. However, bioenergetic studies of glucose transport in N. crassa suggest this is unlikely since glucose symport (glucose transport system II) in N. crassa would be repressed at the concentrations of glucose used in our experiments (Slayman & Slayman, 1974; Scarborough, 1970). The requirement for glucose may instead reflect the necessity for an energy source to provide ATP for the proton pump, which it is presumed drives proton efflux and thereby provides the electrochemical potential for the circulating current.

In order to explain the transcellular current flow reported here it is necessary to suppose that there is a spatial segregating of proton pumps and proton leaks in the hypha. As yet we cannot tell whether leaks are confined to the tips or pumps excluded from it. Experiments are however
underway to attempt to localize the sites of insertion of proton pumping ATPases in the plasmalemma.

The electrical fields generated by cells have been proposed to influence the mobility of charged proteins in the cytoplasm or cell membrane. Polarity is therefore said to result from the electrophoretic or electroosmotic redistribution of morphogenetic proteins in the endogenous electrical field (Jaffe et al., 1974; Jaffe, 1977). Kropf et al. (1983) showed that the current at the tips of branching hyphae of A. bisexualis sometimes reversed transiently from inward to outward, yet hyphal extension continued. Here also, we report outward currents at a few growing hyphae of N. crassa. These reports severely weaken the hypothesis that polarity is a consequence of the electrical fields generated by living cells since growth can clearly continue when the field is reversed. Although the vectorial flux of electrical charge does not seem to correlate well with polarized growth this does not preclude ion currents as important effectors of cell polarity. A series of experiments using pH microelectrodes showed that tip growth of A. bisexualis was invariably associated with proton influx even when hyphae were branching (Gow et al., 1984). Reversal of the direction of electrical current in this organism was therefore thought to be due to fluxes of other ions which masked a continued flow of protons into the tip. If transcellular ion currents are involved in the localization and maintenance of hyphal polarity then it would seem likely that it is the flow of protons (or other ionic species) that is of importance and not the flow of electrical charge.

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REFERENCES


