Transcellular Ion Currents in the Water Mold *Achlya*. Amino Acid Proton Symport as a Mechanism of Current Entry

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ABSTRACT *Achlya*, like other tip-growing organisms, generates an endogenous electrical current such that positive charge flows into the hyphal apex and exits from the trunk. The present study is concerned with the mechanism of current generation by hyphae growing in a defined, complete medium. The intensity of the current, measured in the extracellular medium with a vibrating probe, was unaffected by the removal of all the inorganic constituents of the growth medium. However, an increase in the external pH or the deletion of amino acids abolished the current. Removal of methionine alone diminished the current by two thirds. Hyphae also generated a longitudinal pH gradient in the extracellular medium; the region surrounding the tip was more alkaline than the bulk medium, whereas the region around the trunk was relatively acidic. These findings suggest that a flux of protons, dependent upon amino acids in the medium, carries current into the tip and creates the surrounding alkaline zone.

The proton current appears to result from the transport of amino acids rather than their metabolism. Conditions that abolished the current also inhibited methionine uptake but had little effect on the respiratory rate. The findings imply a connection between the proton current and chemiosmotic energy transduction. We propose that protons flow into the hyphal tip through amino acid/proton symporters that are preferentially localized there. The proton flux energizes the uptake of amino acids into the growing zone and may also contribute to the polarization of hyphal growth.

Growing fungal hyphae deposit new cell wall and plasma membrane only in the terminal region of the tip. The mechanisms that establish this rigorous polarity are pertinent to the spatial ordering of growth and development in general, but are presently quite obscure. In this context, a major clue stems from the discovery by L. F. Jaffe and his colleagues that tip-growing organisms generate transcellular electrical currents. They reported that current (defined as the flow of positive charge) enters the growing tips in germinating zygotes of the brown algae *Fucus* and *Pelvetia* (1, 2), germinating pollen grains (3), and plant roots (4, 5). Remarkably, an inward current often precedes the outgrowth of a new tip and accurately predicts the site of its emergence (2, 3). Such findings have led Jaffe to propose that the transcellular current localizes the site of growth and differentiation (6, 7). We have extended this line of inquiry to the fungi (8, 9), in the belief that these relatively simple organisms may provide further insight into the genesis and functions of transcellular currents.

In the water mold *Achlya*, over a wide range of growth conditions, electrical current enters the tip of a growing hypha and exits from its trunk (9, 10). The present study is concerned with the genesis of the transcellular current by hyphae growing in complete, defined medium. It appears that, under these conditions, the electrical current is carried by protons and results from the spatial segregation of systems that pump protons out of the cytoplasm from those that facilitate their

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return. This conclusion pointed to a connection between the transcellular current and the well-known role of protons in chemiosmotic energy transduction (11–13). In bacteria, fungi, and plants, primary pumps expel protons from the cytoplasm, generating an electrochemical potential gradient with the cytoplasm electronegative and alkaline. The influx of protons across the plasma membrane is obligatorily coupled to the transport of sugars, amino acids, and ions by an array of secondary porters. The free energy of the proton electrochemical gradient is thereby utilized to support the work of nutrient transport. We find that, under our conditions, the proton current of *Achlya* is dependent upon the presence of amino acids, particularly methionine. This leads us to propose that protons are carried into the hypha by electrogenic, secondary symport with amino acids (cotransport) and that the preferential localization of amino acid/H^+ symporters at the tip may be the physical basis of the transcellular proton current in complete medium.

Transcellular electrical currents have also been observed in nutritionally deficient media lacking amino acids (10; Kropf D. L., unpublished results). These can hardly be attributed to amino acid symport, and their nature will be the subject of a future investigation. We would only point out that, if transcellular currents play a fundamental role in growth and development, hyphae must drive currents under a variety of conditions and may well make use of several kinds of transport processes to carry current into the tip.

MATERIALS AND METHODS

Organism and Media: *Achlya bisexualis* T5 was purchased from the American Type Culture Collection; *A. bisexualis* 65-1 was a gift from Dr. Paul Horgen (Department of Botany, Erindale College, Ontario, Canada); and *A. species* was purchased from Carolina Biological Supply Co. (Burlington, NC). Stock cultures of each strain were maintained on PYG agar (Bacto-Peptone, 1.25 g/liter; yeast extract, 1.25 g/liter; glucose, 3 g/liter; and Bacto-Agar, 20 g/liter) and transferred every week. Spores were prepared in $CaCl_2$ according to Griffin and Breuker (14).

The composition of DMA, a complete, defined medium, was as follows: K-PIPES (piperazine-*N*-*N*'-*bis*[2-ethane-sulfonic acid]), 1.0 mM; KH_2PO_4 and K_2HPO_4, 0.5 mM each; glucose, 10 mM. Salts: $MgCl_2$, 1.0 mM; $CaCl_2$, 0.5 mM. Trace metals: H_3BO_3, 11 μM; $MnSO_4$, 1.8 μM; $CoSO_4 \cdot 7H_2O$, 0.7 μM; $NaMoO_4 \cdot 2H_2O$ and $ZnSo_4 \cdot 7H_2O$, 0.4 μM each; $CuSO_4 \cdot 5H_2O$, 0.3 μM. Amino acids: glutamic acid, 1.36 mM; methionine, 0.22 mM; isoleucine, leucine, threonine, valine, and lysine, 0.20 mM each; glycine, arginine, phenylalanine, tyrosine, and serine, 0.10 mM each; histidine, 0.05 mM; tryptophan, 0.02 mM. The pH was adjusted to 6.5 with KOH and KCl was added so that the total K^+ added in this step was 3.0 mM. Deficient DMA, abbreviated dDMA, contained the same concentrations of inorganic salts, trace metals, buffer, and glucose as DMA, but had only two amino acids, methionine (1.0 mM) and glutamate (1.0 mM). dDMA resembled the medium described by Griffin et al. (15). *Achlya* hyphae elongated at a rate of 3–5 μm/min in both DMA and dDMA, but the frequency of branching was reduced in dDMA, probably due to amino acid limitation (16).

Measuring Extracellular Electrical Current: Approximately 100 spores were plated on thin DMA agar plates (11 ml agar medium in a 100-mm petri dish) and incubated overnight at 24°C. A circular punch was used to cut agar plugs (3-mm diam) containing mycelium from the plates; each agar plug was then glued (Silastic Elastomer; Dow Corning, Corp., Midland, MI) to the surface of a cover glass (22-mm diam) in a 100-mm petri dish. Seven cover glasses were placed in each dish and covered with liquid DMA. Within 2 h hyphae began to grow off the edge of the agar into the liquid medium. A short cylinder with grease on its rim was placed over the agar plug and onto the cover glass; the cover glass was then picked up and attached to the bottom of a small recording chamber. The cylinder held medium over the plug and prevented the mycelium from drying out during the transfer. Once the transfer had been completed the cylinder was removed; the cover glass then formed the bottom of the chamber. The circular chamber held 0.9 ml and had two ports for medium exchange; the medium was exchanged using a double syringe (push-pull) apparatus.

The extracellular current map was recorded with a vibrating probe. The operation of this instrument has been described in detail (17, 18); basically, it is designed to detect a potential difference as small as 10 nV between two points in the medium 30 μm apart. Knowing the resistivity of the medium, this voltage is converted to a current by use of Ohm's law. In most experiments the probe was vibrated perpendicular to the hyphal axis so that current entering or leaving the hypha was detected.

Substitution Experiments: To determine whether or not the influx of a particular ion contributes to the current flow, the effects of removing that ion werre investigated. After recording the current pattern of a hypha growing in DMA, 15 ml of DMA lacking the ion of interest were flushed through the chamber. The pertinent ion was replaced by a related one; K^+ was replaced by Na^+, Cl^- by $SO_4^=$ or NO_3^-, and Ca^{++} by Mg^{++}; Na^+, PO_4^{-3} (P_i) and the PIPES buffer were simply omitted rather than being replaced by another ion. To make calcium-free medium, 0.5 mM Mg^{++} was substituted for 0.5 mM Ca^{++}, and EGTA (1.0 mM ethyleneglycol *bis* (β-amino-ethyl-ether)-*N*,*N*'-tetra-acetic acid) was added to reduce the free Ca^{++} concentration even further. The dependence of the transcellular current on glucose and amino acids was determined by analogous substitution experiments.

To check the efficacy of the exchange procedure, the removal of K^+ from the chamber was followed by flame photometry. The first 15-ml flush with K^+-free medium reduced the K^+ concentration from 5.8 mM to 50 μM, the second flush from 50 to 8 μM, and the third from 8 to 3 μM. The K^+ concentration in distilled H_2O was 3 μM. The presence of the small agar plug did not significantly alter the effectiveness of the K^+ washout.

DMA proved unsatisfactory for experiments in which the proton concentration was varied because a precipitate, probably calcium phosphate, formed at pH 7.5 and above. Instead, PYG medium was used; this complex medium was buffered at pH 6.5 or pH 8.5 with PIPES or Tris, respectively.

It took 30 to 40 s to exchange the medium in the chamber, and the movement of liquid past the probe perturbed the voltage recording. By the time reliable measurements could be made, 1 to 2 min had elapsed from the beginning of the exchange. The currents were followed through two, and usually three, exchanges.

Whenever possible, ion replacement was also carried out by growing mycelia on DMA lacking the ion in question. Spores were plated onto 2% DMA agar in which K^+ was replaced by Na^+, or Cl^- by $SO_4^=$. Trace metals or $MgCl_2$ were withheld without replacement. After overnight growth on these solid media, the mycelium was prepared for current measurements as described above.

Two-dimensional Maps: To characterize the pattern of current flow into the growing tip in the horizontal plane, the current was measured both parallel (axial dimension) and perpendicular (radial dimension) to the hyphal surface. The vector pairs measured at the same location were summed to give a single, two dimensional vector.

With the probe vibrating perpendicular to the hyphal surface, the current flowing into the tip in the radial dimension was measured as close to the hyphal surface as possible. (The limits were set by the diameter of the sensing sphere, 28 μm, and by the excursion distance of 30 μm). The probe was then moved away from the surface in 10-μm increments and the decline in the current magnitude was recorded. A series of such measurements were made at positions in front of, beside, and behind the tip.

The microscope stage was then rotated by 90° so that the probe vibrated parallel to the hyphal axis and the axial component of the current was measured at the same positions relative to the tip. Because the magnitude of the current fluctuated during the experiment, both the axial and radial vectors were normalized to the intensity of the initial current flow. This was done by monitoring the current at particular control positions throughout the experiment; the fractional changes in these current magnitudes were used to correct the radial and axial current vectors. The control position in the axial dimension was in front of the tip; in the radial dimension it was 30 μm behind the tip.

Radial Falloff: The decline in magnitude of current flowing perpendicular to the surface was measured in greater detail at a position 60 μm behind the tip, where the axial vector was zero. The probe was vibrated perpendicular to the surface in the same horizontal plane as the hypha. Once this signal was recorded, the probe was moved in horizontal increments away from the hypha, the direction of vibration always remaining perpendicular to the hyphal surface.

Mapping the Extracellular pH: The pH of the medium surrounding a growing hypha was measured at various distances behind the growing tip, and the pH profile along the hyphal length was constructed. This could not be done with hyphae prepared as described above, for the agar block acts as a mild source of acid. Instead, a few spores were inoculated onto sterile polycarbonate filters (8-μm pore size) floating on the surface of DMA medium. The medium rapidly wicked down through the pores, the spores (15-μm diam) remaining above. After overnight incubation at 24°C, hyphae had grown down through the pores into the medium below and were thus trapped on the filters. To measure the extracellular pH, DMA was replaced by a modified medium of

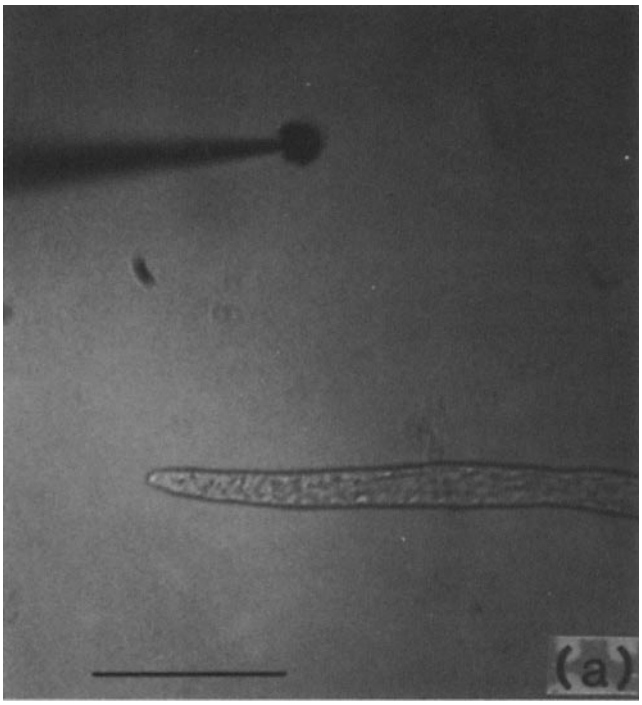
reduced buffering capacity; this lacks both PIPES and P_i and contains only half the normal concentration of amino acids (unbuffered DMA). The filters were first washed by floating them momentarily on this medium. A piece of the filter containing hyphae was then mounted vertically in a chamber containing the unbuffered DMA, pH 6.5. A microelectrode with pH-sensitive glass at its tip (Microelectrodes, Inc. Londonderry, NH) was used to detect the small difference in pH between the bulk medium and the medium directly surrounding a hypha.

Methionine Uptake: The initial rate of methionine uptake was measured at different pH values. Hyphae were grown overnight on polycarbonate filters floating on DMA medium at pH 6.5 and then floated for 3 h on DMA containing 200 μM methionine, but lacking P_i and all other amino acids. Hyphae grew in this medium for hours and generated normal current patterns (data not shown). Immediately before measuring methionine uptake, the mycelia were briefly exposed to the test pH by dipping the filters in media buffered to pH 6.5, 8.5, or 9.5. The buffers were PIPES (piperazine-*N,N'-bis*[2-ethanesulfonic acid], pH 6.5), glycylglycine (pH 8.5), and CHES (cyclohexylaminoethane sulfonic acid, pH 9.5); each was added at a concentration of 15 mM to prevent the pH from drifting during the experiment. The filters were then floated on the same media containing 200 μM [^{14}C]methionine (specific activity, 0.4 mCi/mmol). Individual filters were removed at intervals, washed with nonradioactive medium, and counted. Protein was assayed by the Lowry method (19).

RESULTS

The Pattern of Current Flow

A photomicrograph of the vibrating probe with a hypha of *A. bisexualis* T5 is shown in Fig. 1 *a*. The hypha had grown off the edge of a DMA agar plug into liquid DMA medium; the probe was at a reference position and was not vibrating. When vibrated in the orientation shown, the sensing sphere moved perpendicular to the hyphal axis and therefore measured the radial current vector. Fig. 1 *b* shows a representative chart recording of the current measured at two positions. When the probe was moved from the reference position to the hyphal apex (*A*) the voltage trace was deflected downward, indicating that current flowed into the hypha at this location.



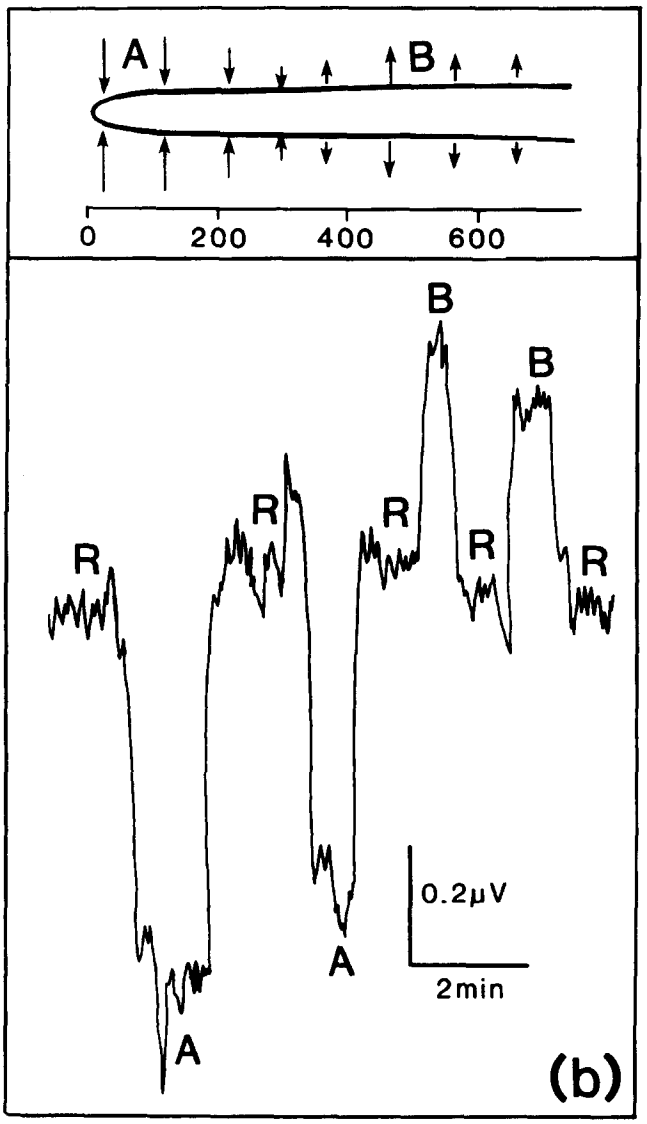


FIGURE 1 (*a*) Micrograph of the vibrating probe used to measure extracellular current flow in the medium surrounding a hypha of *Achlya bisexualis* T5. When this photograph was taken, the probe was at a reference position and was not vibrating. To measure current entering or leaving the hypha, the probe was vibrated close to the cell surface in the orientation shown. (*b*) Representative chart recording. The trace shows the deflection of a voltage recording as the probe was moved from a reference position (*R*), far from the hypha, to a position near the hyphal tip (position *A* in *inset*) or to a position 400 μm behind the tip (position *B*). A downward deflection indicates current entry, while an upward deflection indicates current leaving the hypha. The time constant of the lock-in amplifier was 3 s. Vertical bar, 200 nV; horizontal bar, 2 min. *Inset* depicts the pattern of current flow inferred from many such recordings; the length of each arrow indicates the magnitude of the current at that location. Bar, 100 μm.

At position B, 400 μm behind the tip, the deflection was in the opposite direction, indicating an outward current. The current intensity measured at any one location may vary slowly (by as much as threefold in the course of an hour). The inset portrays the pattern of current flow; current enters over the first 350 μm of the tip and exits from more distal regions. Very similar current patterns were measured along the hyphae of other species of *Achlya* (*A. bisexualis* 65-1 and *A. sp.*).

In most hyphae, the maximal inward current flowing perpendicular to the hyphal axis was found ~30–60 μm behind the tip, well behind the most active growth zone (see Fig. 1 of reference 9). Since this finding appeared to conflict with the hypothesis that the inward current localizes the site of growth, the pattern of current flow around the tip was mapped in greater detail. A two-dimensional map covering the terminal 70 μm was constructed by vector addition; the results are shown in Fig. 2. In front of the tip and over the first 20 μm, current flowed inward and posteriorly; farther back current flowed inward and anteriorly. In between these two regions was a position at which all the current flowed directly into the hypha. In all four tips mapped in detail, the vectors pointed, not toward the apex, but towards a position 20–50 μm behind the tip.

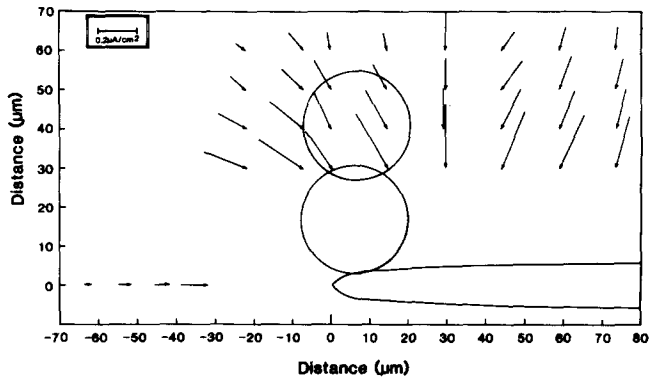


FIGURE 2 Two-dimensional current map. Current vectors in the axial and radial dimensions were summed to give the vectors shown. The midpoint of the probe's sweep was taken as the recording position, and is indicated by the position of the arrowheads; this position was typically 30 μm from the hyphal surface at closest approach. The length and direction of each arrow represents the magnitude and direction of the current flow at that location. The two circles indicate the extremes of the probe's sweep when it was vibrated in the radial dimension at a recording position. The sensing probe and hypha are drawn to scale, emphasizing the limited spatial resolution obtained with this particular electrode. Bar, 0.2 μA/cm^2.

The inward current was characterized further by measuring the radial decay of the current at different distances from the hyphal surface (Fig. 3*a*). Measurements were made at the position where all of the current flowed perpendicular to the surface; this position was 60 μm behind the tip for the hypha examined in Fig. 3. As the probe was moved away from the hypha, the magnitude of the current decreased in a nonlinear fashion. The decay of the current with distance agreed with the decrease predicted by the following model. As a first approximation the hypha was assumed to be a line source of current (abscissa in Fig. 3*b*) with the current strength varying as a function of position along the line. The potential V at a point (z_1, ρ) [Fig. 3*b*] due to a point z on the line is given by

$$V(z_1, \rho) = \frac{1}{4\pi\sigma} \cdot \frac{I(z)}{r},$$

where σ = specific conductivity of the medium, r = distance from the point on the line $(z, 0)$ to the point in space (z_1, ρ) and is $= [\rho^2 + (z - z_1)^2]^{1/2}$, and $I(z)$ = strength of the current at z.

To calculate the radial component of the electric field (E_ρ) we differentiate V with respect to ρ. Finally, to obtain the contribution from all points on the line, the expression for E_ρ is integrated over z.

$$E_\rho(z_1,\rho) = \frac{\rho}{4\pi\sigma} \int \frac{I(z)}{[\rho^2 + (z - z_1)^2]^{3/2}}\, dz.$$

If we assume that the current strength, $I(z)$, varies linearly with z (see Fig. 3*c*), then E_ρ can be integrated analytically for each of the four regions ($z = 0$ to $z = z_1$, $z = z_1$ to $z = z_2$, etc.). The continuous curve in Fig. 3*a* was calculated from this analysis and fit to the data (since the electric field is proportional to the current). The curve was normalized to the experimental data by a single scaling factor. The closest measurement to the hyphal surface was 20 μm and the radius of the hypha was 5 μm. When the curve is extrapolated to the surface of the hypha, we estimate that the density of current

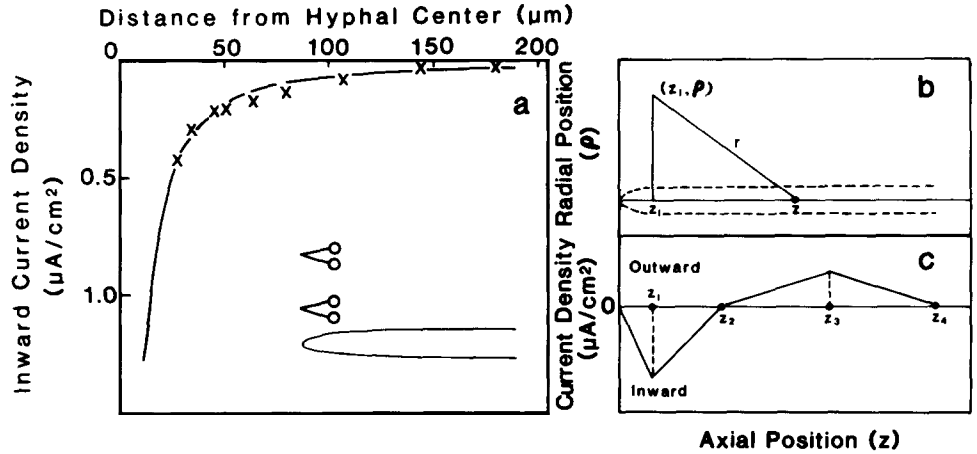


FIGURE 3 Radial decay of current density. (*a*) Measurements of current density (x) at a fixed position behind the tip (60 μm) where the inward current was maximal. The probe was moved away from the hypha in increments as shown in the inset (not to scale). The solid curve was fit to the data as described in the text. By convention, inward current is plotted below the abscissa. (*b*) Coordinate system used to calculate the voltage and electric field at a point in space due to a point source of current (at position z) on a line. The dotted line indicates the surface of the hypha with its tip at the origin. The line source of current is assumed to be at the center of the hypha. (*c*) Current strength as a function position along the hyphal axis. The triangular shapes of the inward and outward current are close approximations to the actual current patterns measured. The curve in Fig. 3*a* was calculated with $z_1 = 60$ μm, $z_2 = 300$ μm, $z_3 = 600$ μm, and $z_4 = 900$ μm.

crossing the plasma membrane is 2.5 $\mu A/cm^2$. This varied from 0.5 to 2.5 $\mu A/cm^2$ for different hyphae.

The Effects of Ion Substitution

If the influx of a particular ion were responsible for part of the electrical current, removal of that ion from the medium should result in a marked decrease in the magnitude of the current flow. These experiments were conducted in two ways. First, medium exchange was used to subject hyphae grown on complete DMA to acute reductions in extracellular ion concentrations; a single medium exchange decreased the concentration of the pertinent ion 100-fold within seconds. The current pattern was unaffected when any one of the following ions was removed in this way: K^+, Cl^-, Na^+, P_i, or the PIPES buffer. For example, Fig. 4*a* shows the current pattern generated by a hypha grown on DMA and then flushed twice with DMA in which all the Cl^- was replaced by $SO_4^=$; removal of over 99% of the Cl^- did not affect the current pattern.

To rule out particular ions more rigorously, a mycelium was grown from spores on DMA agar plates in which the ion replacement had already been made. Currents were recorded in the deficient media; in the experiments reported below, hyphae grew at the normal rate of 4–5 μm/min and appeared perfectly normal. Hyphae grown on K^+-free DMA (Fig. 4*b*), Mg^{++}-free DMA (Fig. 4*c*), or on DMA lacking trace metals (Fig. 4*d*), generated current patterns that were well within the normal range. (As the trace-metal mixture was the only source of Na^+ in DMA medium, Na^+ ions are also not required). Similar results were obtained with hyphae grown on Cl^--free DMA (data not shown). *Achlya* grew poorly on media lacking P_i or Ca^{++} ions; these could only be examined by acute removal. Taken together, these ion-substitution experiments showed that the current was not dependent upon external K^+, Na^+, P_i, PIPES, Cl^-, Mg^{++}, or trace metals; only Ca^{++} and H^+ remained as likely candidates.

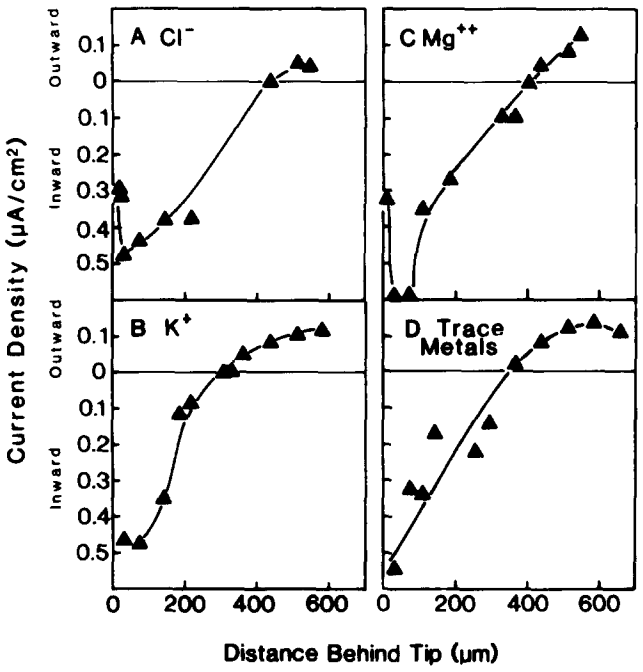


FIGURE 4 Current patterns generated by hyphae in ion-substituted DMA. The ordinate gives current densities measured with the probe vibrating in the radial dimension. Each point represents the current density measured at a known distance behind the tip (indicated on the abscissa). *a* represents a hypha subjected to acute removal of Cl^-; *b*, *c*, and *d* depict hyphae grown on media depleted of one or more ions. (*a*) Cl^- was removed and replaced with $SO_4^=$ by medium exchange. This current pattern was recorded after two exchanges for the Cl^--free DMA. (*b*) This hypha was grown on, and extracellular currents measured in DMA in which the K^+ was replaced by Na^+. (*c*) Procedure as in *b* except that the medium was DMA lacking Mg^{++}. (*d*) Procedure as in *b* except that the medium was DMA lacking trace metals.

Effects of Calcium Removal

The effects of Ca^{++} removal were complex. Zoospores failed to germinate on a medium in which Ca^{++} was replaced by Mg^{++} plus 1 mM EGTA. During acute Ca^{++} removal, the free Ca^{++} concentration was reduced by ~5 orders of magnitude, (from 0.5 mM to 5 nM; see reference 20) by exchanging DMA

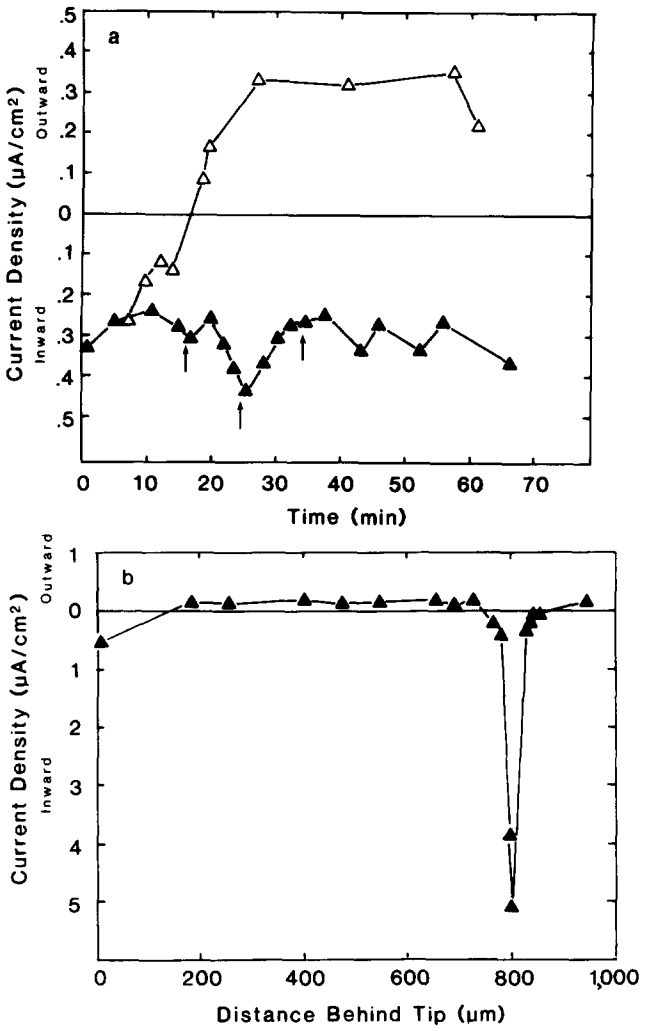


FIGURE 5 (*a*) Acute calcium removal. The current 30 μm behind the tip was monitored on two hyphae 500 μm apart in the same dish. The hyphae were grown on DMA. At the time indicated by the first arrow, this medium was exchanged for Ca^{++}-free DMA, in which all the Ca^{++} was replaced by Mg^{++}. 1 mM EGTA was also present to chelate any remaining Ca^{++}. The hyphae were subsequently exposed to two additional flushes with the Ca^{++}-free medium (second and third arrows). (*b*) Current pattern after long-term Ca^{++} starvation. After 2.5 h in the Ca^{++}-free DMA, current flow was markedly altered; nearly all of the current entered a narrow zone far behind the hyphal tip and exited over the remainder of the hyphal surface.

for the Ca^{++}-free medium containing Mg^{++} and EGTA. Ca^{++} removal eventually caused the inward current at the tip to turn outward, but this response was often delayed, as shown in Fig. 5*a*. In this experiment, we followed two hyphae a few hundred micrometers apart in the same dish. When DMA was exchanged for the Ca^{++}-free medium, the inward current in one tip turned outward within minutes; in contrast, the neighboring hypha continued to drive normal currents for the duration of the experiment (approximately another 50 min), including two additional flushes with the Ca^{++}-free medium. Similar experiments were done on a total of 16 hyphae, of which 11 showed no immediate response to Ca^{++} deprivation. In all 16 hyphae, the apical current eventually did turn outward, and growth ceased. Nonetheless, in the short term, the bulk of the inward current was not dependent upon external Ca^{++}. It is difficult to assess the degree to which calcium influx contributes to the inward current; given the fluctuations in current intensity, a Ca^{++} contribution of 10% or less would have gone undetected.

Remarkably, when the current at the tip turned outward in the absence of Ca^{2+}, a very large and highly localized inward current appeared some distance behind the tip (Fig. 5*b*). This peak of inward current was associated with a visible structural change in the cytoplasm at that location; the cytoplasm appeared more vacuolated and refractile than normal, but this observation was not pursued further. When Ca^{++} was added back to the bathing solution by medium exchange, the normal current pattern recovered: within 15 min, the peak of inward current reversed to a small outward current; simultaneously, inward current reappeared at the tip (data not shown).

Evidence that Protons Carry the Inward Current

Hyphae in PYG buffered to pH 6.5 generated an inward current of normal magnitude at the tip (Fig. 6). When this medium was exchanged for PYG buffered at pH 8.5, the inward current began to decrease even before the first measurement could be made. The inward current continued to drop and turned outward at the tip within minutes. As long as the pH was maintained at 8.5 the current pattern was reversed, i.e., current left the tip and entered more distal regions. However, the spatial distribution of the current varied with time. None of the hyphae grew at pH 8.5. When the pH was returned to 6.5, inward current quicky reappeared at the tip and growth resumed.

The dependence of the inward current upon the external pH suggests that protons carry the charge influx. If the current were due primarily to proton fluxes, one would expect to find the medium alkaline near the tip and acidic farther back. To test this possibility, we measured the extracellular pH along the length of hyphae growing in unbuffered DMA as described in Materials and Methods; hyphae grew for hours in this phosphate-free medium and generated normal current patterns. Fig. 7 shows the pH profile observed under these conditions. At the tip and extending 200 μm beyond, the pH adjacent to the hypha was slightly alkaline with respect to the bulk phase; the pH turned slightly acid farther back. The acidic region extended over the entire hypha, except for the 200 μm region at the tip.

A rough calculation suggests that the intensity of the proton current is adequate to account for the alkaline zone surrounding the tip. To estimate the rate at which protons diffuse from the bulk medium (acidic) to the medium near the hyphal tip (alkaline), we assumed steady state conditions for proton

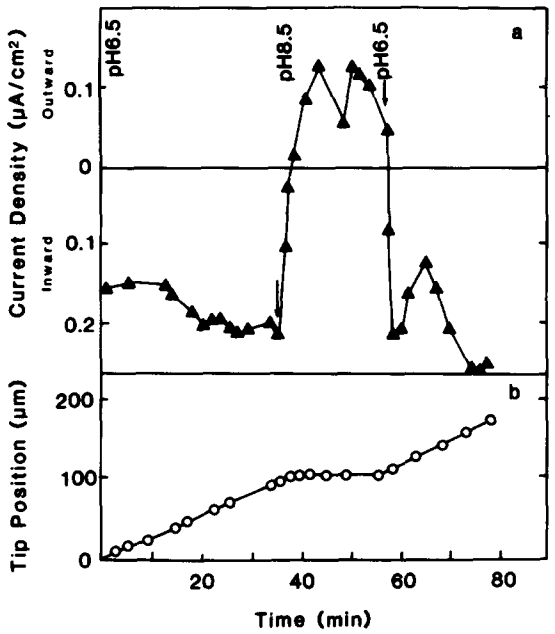


FIGURE 6 Dependence of the inward current upon extracellular pH. (*a*) The current density 30 μm behind the tip was monitored in PYG medium. The pH of the bathing medium was increased from 6.5 to 8.5 by medium exchange (first arrow), and then lowered again to 6.5 (second arrow). Current flow into the tip was abolished at pH 8.5. (*b*) Growth. The hypha grew at pH 6.5 but not at pH 8.5.

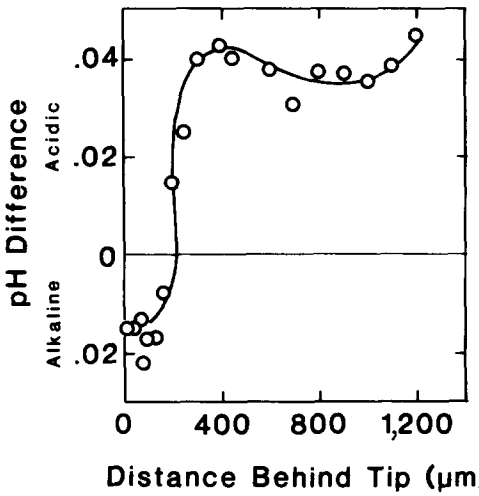


FIGURE 7 pH profile of a growing hypha in unbuffered DMA. Each point represents the difference in pH between the bulk medium and the medium just outside the hyphal wall. To make each recording, the pH electrode was moved from a reference position to a position where the pH glass nearly touched the hyphal surface; once the voltage change was recorded the electrode was returned to a reference position. As the pH-sensing glass at the electrode tip was 50 μm long, the data represent the average pH of a substantial amount of medium.

diffusion into a hollow cylinder in which the external pH is 6.50 (pH of bulk medium) and the internal pH is 6.52 (average pH measured near the hyphal tip, see Fig. 7). The concentration profile of protons in the medium within the cylinder is given by the logarithmic function $C = A + B \ln r$, where C is concentration, r is the radius of the cylinder and A and B are constants determined by boundary conditions (21). Differen-

tiating this function with respect to r, and using Fick's law, we calculate that protons diffuse to the hyphal surface at a rate of 1.07×10^{-4} picomoles per second. These protons must enter the hypha over the terminal 200 μm and would generate a current density at the plasma membrane of 0.32 μA/cm^2. The current measured with the vibrating probe extrapolates to 0.5 to 2.5 μA/cm^2 at the hyphal surface (Fig. 3), which is sufficient to support the alkalinization actually observed.

The Proton Current Requires Exogenous Amino Acids

In fungi, the transport of organic nutrients is often coupled to proton influx (22). The removal of glucose from DMA had no effect upon the inward current at the tip; likewise, hyphae grown on glucose-free DMA generated normal current patterns (data not shown). In contrast, when DMA was exchanged for DMA lacking amino acids, the inward current at the tip disappeared even before the first measurement could be made (Fig. 8). The hypha ceased to elongate and the current at the tip remained outward for as long as amino acids were withheld from the medium. Readdition of amino acids relieved the inhibition; the hypha resumed growth and inward current returned to the tip.

DMA contains 14 amino acids, making it difficult to identify those that were required to support the inward current. Instead, the experiments were done in dDMA medium whose amino acid supplement consisted only of 1.0 mM methionine and 1.0 mM glutamate. Hyphae in dDMA generated the same pattern of current flow as in DMA. The effect of removing either methionine or glutamate from dDMA is shown in Fig. 9. When glutamate was removed by three successive flushes with glutamate-free medium, the magnitude of the inward current was not significantly altered. In contrast, the inward current was immediately abolished when methionine was removed. Likewise, current flow into the tip resumed when

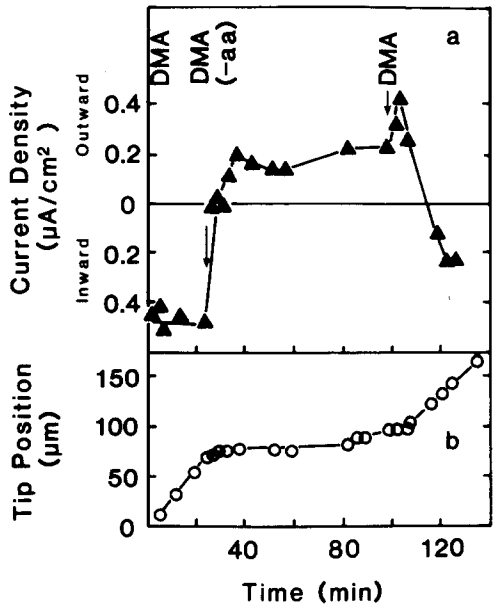


FIGURE 8 Dependence of the inward current upon exogenous amino acids. (*a*) The current was monitored 30 μm behind the tip as DMA medium was replaced by DMA minus amino acids (denoted DMA (−*aa*), first arrow). At the time indicated by the second arrow, DMA was restored to the chamber. (*b*) Growth, like current flow, ceased upon amino acid removal.

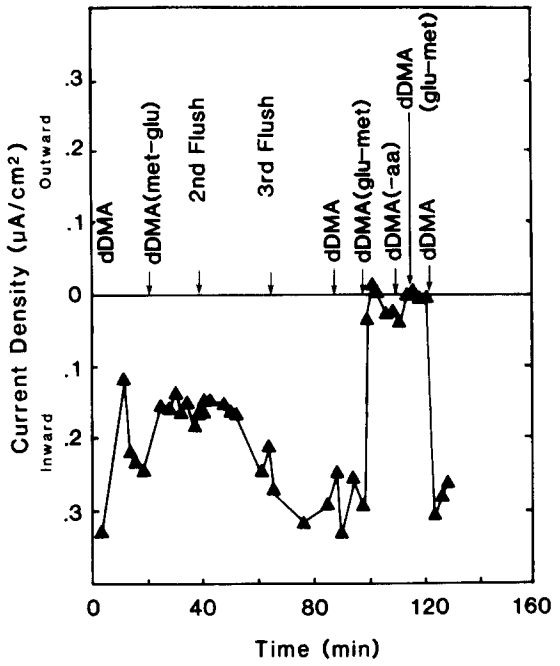


FIGURE 9 Dependence of the inward current upon methionine and glutamate in dDMA. Although similar to the previous figure, this figure is more complex. In dDMA the normal inward current was found near the tip; when dDMA was exchanged for dDMA minus glutamate (denoted *dDMA(met-glu)*) a small drop in inward current was recorded (first arrow). However, the inward current gradually increased in magnitude during two subsequent flushes with dDMA(met-glu) (second and third arrows). Adding back the glutamate by medium exchange for dDMA did not increase the current (fourth arrow). In contrast, when dDMA was exchanged for dDMA minus methionine (denoted *dDMA(glu-met)*) the inward current disappeared before a measurement could be made (fifth arrow). Subsequent removal of glutamate by flushing with dDMA minus amino acids (*dDMA(−aa)*) had no effect (sixth arrow). The glutamate and methionine were then successively added back by flushing with dDMA(glu-met) (seventh arrow) and then dDMA (eighth arrow). Only methionine addition caused inward current to reappear at the tip. Additional experiments showed that the order in which these amino acids were removed or added did not affect the results. The hypha grew in every medium except dDMA(−aa).

methionine was reintroduced to the medium; the readdition of glutamate did not support the inward current.

Methionine also supported most of the inward current in DMA medium; the magnitude of the inward current fell by two thirds when methionine alone was removed from DMA (data not shown). It follows that the remainder of the current was dependent upon one or more of the other 13 amino acids in DMA. Preliminary results suggest that a small fraction of this current was dependent upon leucine (data not shown); the others have yet to be identified. For present purposes, the key finding is that the inward current in both DMA and dDMA represents amino acid-dependent proton influx, with methionine sustaining the majority of the current.

We have previously shown that an inward current precedes branching in *Achlya* and accurately predicts the site of branch emergence (9). To investigate whether this current can also be sustained by protons plus amino acids, hyphae were placed in a minimal medium containing methionine and calcium ions, buffered at pH 6.5 and osmotically balanced with mannitol (calcium ions are required to maintain growth). Hyphae continued to elongate for hours in this solution; they gener-

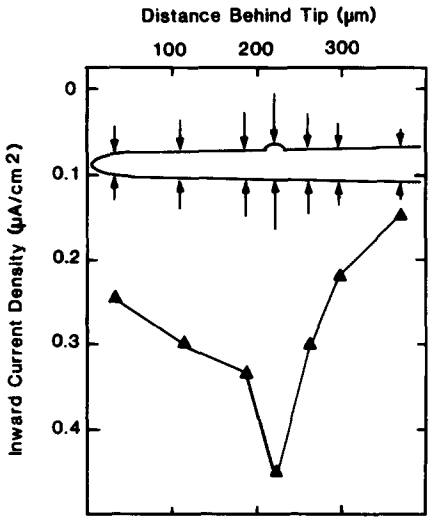


FIGURE 10 Inward current precedes and predicts branching in a minimal medium. This medium contained 1.0 mM methionine, 0.5 mM $Ca(OH)_2$, and 24 mM mannitol as an osmoticum; 1 mM PIPES was added to adjust the pH to 6.5. DMA in the recording chamber was replaced by the minimal medium and the spatial distribution of the current was monitored. This current pattern was recorded half an hour after the exchange while a branch emerged 220 μm behind the tip (see *inset*). The inset depicts the inward current concentrated at the branch site.

ated normal current patterns, except that the region of inward current was confined to the terminal 50 to 100 μm. (As expected, the inward current at the tip disappeared when methioine was removed [data not shown]). Despite the austerity of the calcium/methionine medium a few hyphae branched; when this occurred, a new peak of inward current predicted the position of the nascent branch. Fig. 10 shows an example; the position of maximal inward current was over 200 μm behind the tip and coincided with the branch point. In this medium, unbranched hyphae normally had outward current at this position. It should be mentioned that, having formed, the branches degenerated, presumably because of the lack of sufficient nutrients. Even though the branches ceased to grow, inward current did precede and predict branching in a medium containing only methionine, Ca^{++} and protons; suggesting that this current also results from methionine-dependent proton influx.

As a check on the preceding experiments, we investigated the possibility that alkaline pH or amino acid deprivation brought about a general inhibition of metabolic activity. This is clearly not the case. The $Q[O_2]$ of young mycelia growing in PYG medium at pH 6.5 was typically 400 μl O_2/h mg protein. When the pH was then increased to 8.5, oxygen consumption dropped by an average of 30% (range = 14 to 48%). Similarly, germlings grown in DMA and then resuspended in DMA lacking both glucose and amino acids continued to respire at half the rate of germlings resuspended in DMA (data not shown). Manavathu and des S. Thomas reported earlier that *Achyla* respired for hours in the absence of exogenous carbon and energy sources (23), presumably at the expense of intracellular storage materials. Addition of amino acids to DMA devoid of a carbon source stimulated respiration only nominally: 1.0 mM methionine increased the $Q[O_2]$ from 190 to 200; 10.0 mM glutamate failed to stimulate oxygen consumption (240 vs. 240) and the complete amino acid mixture plus glucose increased the respiratory rate from

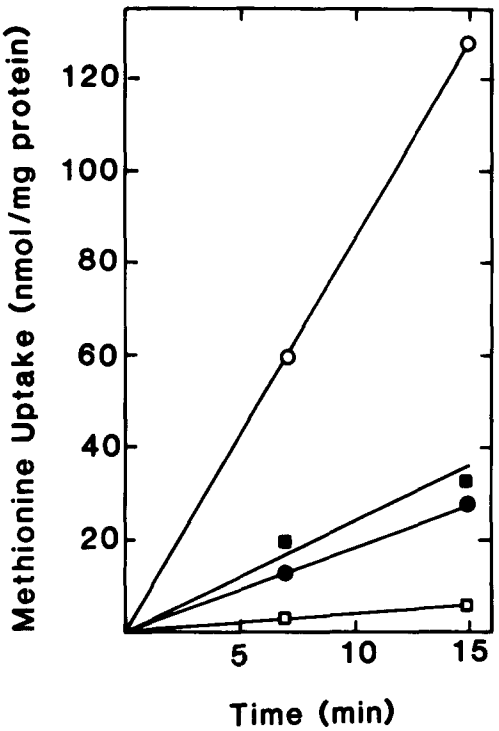


FIGURE 11 Initial rate of methioine uptake at different pH values. Hyphae, grown on filters as described in Materials and Methods, were exposed to radioactive methionine for either 7 or 15 min at pH 6.5 (○), 8.5 (●), or 9.5 (□). Uptake into energy depleted mycelium at pH 6.5 was measured in presence of 10 μM antimycin A (■). Each point represents an average of four filters.

210 to 260. Thus, unlike the transcellular current, respiration was not critically dependent upon exogenous amino acids or the pH of the medium.

Methionine Uptake

Of all the nutrients in DMA, the inward current was affected only by changes in pH and amino acid concentration, suggesting that the symport of protons with amino acids carries the current. If this were true, conditions that inhibit current flow should inhibit amino acid uptake. We therefore measured the rate of methionine uptake as a function of the pH (Fig. 11). The initial rate of methionine uptake was fivefold greater at pH 6.5 than at pH 8.5, and nearly 20-fold greater than that at pH 9.5; similar findings were reported by LéJohn and Stevenson (24). Methionine uptake at pH 6.5 was an energy-requiring process, as judged by the inhibitory effects of Antimycin A, an inhibitor of mitochondrial respiration. Thus, both methionine uptake and the inward current are inhibited at alkaline pH.

DISCUSSION

How the Transcellular Current is Generated

Transcellular ion currents have been described in diverse organisms from molds to man, but the transport systems that carry the current across the plasma membrane remain elusive. We report here that the water mold *Achlya* drives a current of protons through itself. In complete medium, these protons appear to enter the hyphal tip via a transport mechanism of major physiological importance, namely, by symport with amino acids.

The conclusion that protons carry the bulk of the current into the hyphal tip rests on three lines of evidence. (*a*) The electrical current persisted despite the deletion of all other ions from the medium. Even calcium ions, which are clearly required for *Achlya* to grow, do not make a sufficiently large contribution to be detected by our methods; we estimate that Ca^{++} cannot carry more than a tenth of the current. (*b*) The current was reversibly abolished when the pH of the medium was raised to 8.5. (*c*) Growing hyphae generated an extracellular pH profile, with the medium adjacent to the tip slightly alkaline compared with the bulk phase. It should be noted that we cannot distinguish a flux of protons in one direction from a flux of bicarbonate or hydroxyl ions in the other. Analogous arguments led Weisenseel et al. (4) to conclude that growing barley roots drive a transcellular proton current.

A more novel conclusion drawn from the present studies is that the protons that carry current into the hyphal tip in DMA cross the plasma membrane by symport with amino acids. We base this hypothesis on the following observations. (*a*) The proton current was reversibly abolished when amino acids were withdrawn from the medium. Several amino acids contribute, but methionine supports the majority of the current in our media. (*b*) When the pH of the medium was raised to 8.5, current and methionine uptake were inhibited in parallel, but respiration was little altered; this suggests that the transport of methionine across the plasma membrane is coupled to the flow of current. (*c*) Current flow was sensitive only to changes in amino acids and protons; removal of glucose, or of the ionic constituents of the medium, was without effect. The eventual cessation of current flow upon calcium deprivation may reflect the dependence of methionine uptake on Ca^{++} as reported by Singh and LéJohn (25). (*d*) Our most recent data show that the addition of 1 mM methionine to DMA medium devoid of a carbon source causes the membrane potential of *Achlya* to depolarize rapidly by 60 to 100 mV (not shown). Thus, methionine transport appears to be an electrogenic process. The simplest explanation of these findings is that amino acids are taken into the hyphal tip by symport (co-transport) with protons, a mechanism well documented in fungi (22). We recognize, however, that other conceivable mechanisms of proton entry, such as an amino acid-activated proton channel, have not been ruled out.

We are less confident of the ionic composition of the outward current, but here again the evidence points to protons. Ion-substitution experiments argue against anion influx. Cation efflux is difficult to study since we cannot easily manipulate intracellular cation concentrations. Nonetheless, the production of outward current by hyphae grown in the absence of added K^+, Mg^{++}, or Na^+ suggests that the cations do not contribute to this current. Direct, if tenuous, evidence that outward current is carried by protons comes from the fact that regions of outward current are also acidic. However, since the acid region extends far beyond the zone of outward current, it is likely that metabolic products also contribute to the acidity.

The working hypothesis that emerges from the preceding argument is depicted in Fig. 12. We propose that symporters for methionine and other amino acids are concentrated at the hyphal tip and at a new branch. Simultaneous translocation of methionine and protons by the symporters generates the local inward current; if either substrate is removed from the medium, current ceases immediately. The genesis of the outward current is uncertain; we believe that these protons are ejected by an electrogenic ATPase, but experimental evidence is still slender. There is ample evidence for both nutrient/proton symport and proton-translocating ATPases in fungi. For example, extensive studies with *Neurospora* (26–28) and yeasts (29–31) have shown that an electrogenic ATPase ejects protons, thereby creating a large electrochemical proton gradient directed inward. The influx of protons, down their electrochemical gradient, drives the uptake of amino acids (22) and of glucose (32, 33). All that we add to this conventional view is the proposal that the spatial separation of symporters from pumps generates a transcellular proton current.

The actual distribution of symporters and ATPases is not known: symporters may be concentrated at the tip, ATPases may be excluded from the tip, or both. The arrangement depicted in Fig. 12 is consistent with what we know and would permit the hypha to transport amino acids (major metabolic substrates) preferentially into the active growth zone. However, we do not wish to imply that pumps and porters are completely segregated; a small spatial imbalance would suffice to generate the proton current. If we assume that the plasma membrane of *Achlya* pumps out protons electrogenically at the same rate as that of *Neurospora*, 20 $\mu A/cm^2$ (26), then the measured transcellular current, on the order of 1 $\mu A/cm^2$, would comprise just 5% of the total electrogenic proton flux. Spatial overlap between pumps and porters, varying somewhat as the hypha elongates, may also explain why the current patterns differ subtly from one hypha to another (Fig. 4) and fluctuate in intensity as the hypha elongates.

A radical alternative to the interpretation developed above

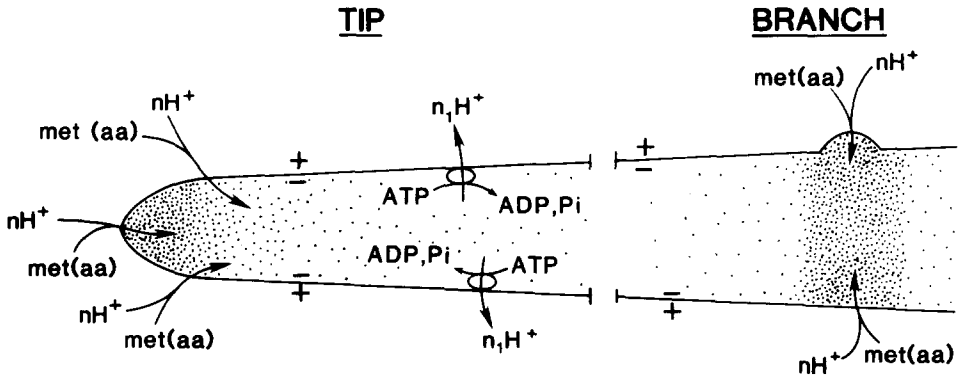


FIGURE 12 Model proposed to account for the generation of transcellular ion currents by *A. bisexualis* T5 growing in DMA medium. The inward current at the tip is postulated to result from the symport of *n* protons with one methionine molecule through porters concentrated at the hyphal tip. The distribution of these symporters is uniform around the hyphal circumference. The outward current is thought to be generated by an electrogenic ATPase that extrudes n_1 protons per ATP hydrolyzed. The stippling inside the hyphal tip depicts a proton concentration gradient that may help maintain polarized tip growth. The inward current at a future branch site also reflects met/H^+ symport.

(Fig. 12) is that current entry into the tip somehow results from elongation of the hypha, and is not directly related to amino acid transport at all. Inward current would cease when methionine is withdrawn because elongation stops, not because symport is blocked; by the same token, current would cease when the pH is raised because elongation halts, not because proton-linked symport is prevented. We discount this hypothesis because there are several situations in which the inward current can be dissociated from top elongation; hyphae have been seen to drive currents even though they are not growing, and have been seen to grow in the absence of electrical current flow. One such example is described in Fig. 9; the hypha elongated in dDMA (glu-met) but did not generate a current. Clearly there is no mutual dependence between the flow of electrical current into the tip and hyphal elongation. Nevertheless, we expect to find a more subtle connection between them, since the spatial separation of pumps from leaks will probably be maintained by continued growth.

Transcellular Currents and Polarity

It is an attractive hypothesis that transcellular ionic currents, which are particularly prominent in tip-growing organisms, play a fundamental role in the genesis and maintenance of polarity. Calcium currents, in particular, have been assigned this role: calcium ions are required for wall growth by exocytosis, and local calcium fluxes have been implicated in polarizing out-growth in a number of plant systems (34–37). *Achlya,* likewise, requires Ca^{++} ions for zoospore germination and for sustained growth; furthermore, membrane-bound calcium accumulates at the hyphal apex (38). Some calcium influx may persist even in the presence of EGTA, since there is an electrical potential of −160 mV (unpublished data) across the plasma membrane; we maintain only that any such calcium flux probably comprises less than a tenth of the transcellular current. We would, however, draw attention to observations that implicate protons in polarized growth. For example, Turian has reported that hyphal tips, nascent branches and buds, even the point of outgrowth of germinating pollen grains, are strikingly acidic (39–41). In our hands, proton-conducting ionophores make *Achlya* branch (Harold, R. L. unpublished results) and they also polarize the germination of *Fucus* zygotes (42). Local acidity may affect the differential assembly of the cytoskeleton, either directly (43, 44) or via the release of calcium from membrane-bound stores, and thus contribute to the process of polarization.

In this context, it was disturbing to find that in *Achlya* the inward proton current is dispersed over the terminal 350 μm, with the point of maximal influx some 30 to 60 μm behind the tip (Fig. 2), while new cell wall is deposited chiefly in the apical 4 μm (45). However, this finding may be deceptive. Growing tips are tapered throughout the apical region; our measurements show that, on the average, the diameter of an *Achlya* hypha increases nearly threefold over the first 30 μm. Thus, the inward current density at the plasma membrane may well be maximal at the apex when one takes into account the small surface area. Moreover, the consequences of current entry into the cell will be magnified near the tip because of its relatively small internal volume. There is, therefore, no conflict between the current map and a possible role for the transcellular proton current in the localization of growth.

In the media used here, protons appear to enter the growing tip by symport with amino acids, but this cannot be the only mechanism of current generation available to *Achlya*. According to Armbruster and Weisenseel (10), hyphae grown on hemp seed in distilled water drive a transcellular current with the polarity described above, yet their medium cannot contain more than trace amounts of amino acids. We, also, have found that *Achlya* can adapt to medium lacking methionine over a period of 1–2 h, resume growth and recover inward current at the tip. Since the pH-profile also recovers, we believe that a proton current is again indicated. These preliminary observations reinforce our suspicion that the transhyphal ion current is not merely a secondary consequence of amino acid transport, but one link in the chain of signals by which hyphae impose direction upon their own growth. If this is true, fungi need a variety of ways to ensure that, so long as a hypha grows, protons flow into its tip.

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