Cytosolic pH Gradients Associated with Tip Growth

Bryan C. Gibbon and Darryl L. Kropf*

The presence of a cytosolic pH gradient and its relation to polar tip growth was investigated in rhizoid cells of *Pelvetia* embryos with the use of pH-sensitive microelectrodes and by ratio imaging. Growing rhizoid cells generated a longitudinal pH gradient in which the apical cytosol was 0.3 to 0.5 units more acidic than the cytosol at the base of the cell. Treatment with a membrane-permeant weak acid, propionic acid, dissipated the cytosolic pH gradient and inhibited growth. The magnitude of the pH gradient correlated well with the rate of tip elongation. The pH gradient spatially superimposed on the cytosolic calcium gradient, and inhibition of calcium fluxes by treatment with lanthanum abolished the pH gradient and inhibited growth.

Tip growth is a common form of cell extension in eukaryotes; examples include growth of neurons, pollen tubes, fungal hyphae, and algal filaments. This growth form serves as a paradigm for cell polarity because cell extension is restricted to a narrow zone at the apex. Specific molecules, organelles, and physiological processes are localized to defined zones along the length of the cell. This regional cytoplasmic differentiation is thought to be regulated in part by cytosolic ion gradients, specifically Ca^{2+} or H^+ gradients (*1*). Cytosolic Ca^{2+} gradients have been measured in many different tip-growing cells and are important regulators of cell extension (*2, 3*). Less is known about pH gradients: Cytosolic pH gradients have been visualized with dyes (*4, 5*), but their function in tip growth has not been investigated.

We have conducted a quantitative analysis of cytosolic pH gradients in tip-growing rhizoid cells of the brown alga, *Pelvetia fastigiata*. *Pelvetia* zygotes acquire and express polarity early in development (*6*). At the two-celled stage, embryos are comprised of a tip-growing rhizoid cell and an isodiametrically expanding thallus cell, which give rise to holdfast and fronds, respectively. Previous observations have implicated pH gradients in polarity establishment and rhizoid growth. When incubated in an applied, external pH gradient, zygotes polarize in accordance with the gradient and rhizoids grow toward the source of acid (*7*). The ability to sense and respond to an imposed pH gradient may be related to the finding that zygotes and young embryos generate external pH gradients in which the rhizoid position is relatively alkaline (*8*).

We investigated cytosolic pH (pH_c) gradients by measuring pH_c with microelectrodes at various positions along the growing rhizoid cell (Fig. 1A). The sensitivity, low noise, and reproducibility of pH-selective microelectrodes permitted detection of differences in pH_c between the apical and basal cytoplasms (Fig. 1B and Table 1). Measurements in the apical 10 μm of the rhizoid cell clustered around pH 7.15 (proton activity = 71 nM), whereas those made beyond 40 μm from the tip averaged pH 7.45 (proton activity = 35 nM). In the region between 10 and 40 μm, there was a nearly linear rise in pH_c with increasing distance behind the tip (Fig. 1C).

Because a cytosolic voltage gradient would interfere with the accuracy of pH_c measurements, the membrane potential (V_m) profile along the rhizoid length was measured. No significant differences in V_m were detected (Fig. 1D), indicating that the rhizoid cytoplasm was isopotential. We ruled out the possibility that microelectrode impalement caused differential membrane damage at the tip and base by measuring input resistances; values at the tip [14.2 ± 3.6 megohms (n = 6)] and base [13.5 ± 7.3 megohms (n = 5)] were not significantly different.

We confirmed the presence of a cytosolic pH gradient by microinjecting the dual emission probe SNARF1 conjugated to dextran (molecular weight, 10,000) into fertilized eggs and imaging rhizoid pH profiles a day later (two-celled stage) by confocal microscopy. Initial results have identified a pH gradient of similar orientation and magnitude to that detected by electrophysiological techniques (Fig. 2).

A cytosolic gradient of protons necessarily results in a flux of protons from tip to base in the cytoplasm, and the magnitude of the proton flux can be calculated with the use of diffusion models (*9*). We calculate that the measured gradient would drive a cytoplasmic proton current of approximately 3×10^{-6} μA and that this flux would tend to dissipate the gradient. In order to maintain a steady-state gradient, there must be spatial segregation of proton

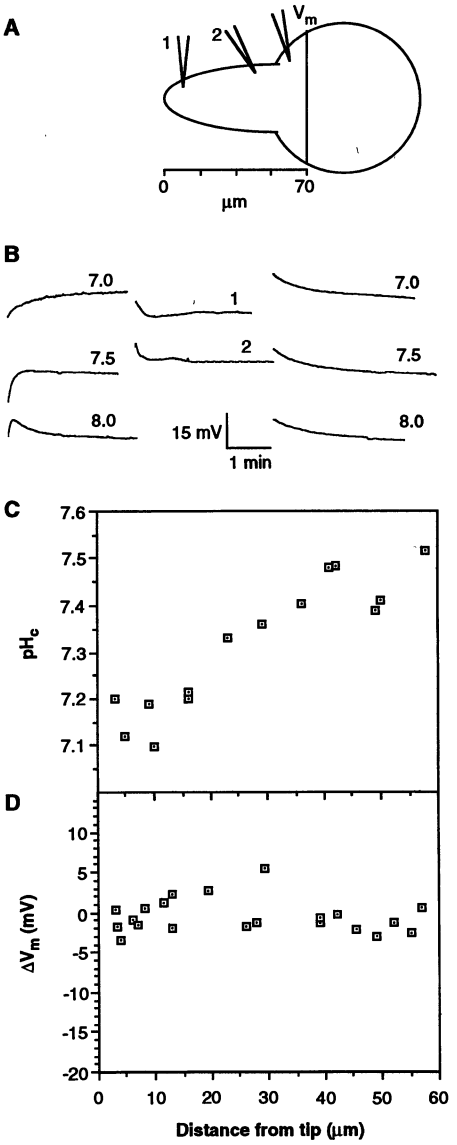

Fig. 1. Measurement of pH_c. (**A**) Microelectrode placement. Zygotes were grown in ASW (pH 8.2) to the two-celled stage (24 to 28 hours after fertilization). A voltage microelectrode filled with 3 M KCl was inserted near the base of the rhizoid cell, and a turgor-resistant, pH-sensitive microelectrode was inserted at the desired recording position. Two recording positions (1 and 2) are shown. Only one measurement was made per cell. Turgor-resistant, pH-sensitive microelectrodes were fabricated, calibrated, and used as previously described (*8, 22*). (**B**) Calibration and measurement. The calibration on the left corresponds to recording 1 (pH_c = 7.10), and the calibration on the right corresponds to recording 2 (pH_c = 7.44). (**C**) The pH_c profile. The abscissa indicates the position of the pH-sensitive microelectrode. V_m was typically −55 to −80 mV. (**D**) V_m profile. Microelectrode placement was as described above except that the pH-sensitive microelectrode was replaced with a second voltage microelectrode. The difference recording (ΔV_m) is plotted against the position of the more apical microelectrode.

Department of Biology, University of Utah, Salt Lake City, UT 84112, USA.

*To whom correspondence should be addressed.

sources and sinks. In principle, a source of cytoplasmic protons could be provided by proton influx across the plasma membrane (*10*), but bioenergetic coupling to protons is unlikely in *Pelvetia* because the proton motive force is small (*11*). Instead, protons produced by metabolism probably contribute to cytoplasmic acidification. For a steady-state gradient to be maintained, protons must be consumed at, or pumped from, the base of the rhizoid. If protons are pumped out across the basal plasma membrane, a current density of 49 nA cm^{-2} over the basal 30 μm of plasma membrane would be sufficient to maintain the measured cytosolic pH gradient. This localized proton current would be only a small fraction (<5%) of the total electrical current that exits the base of the rhizoid cell (*12*). *Pelvetia* zygotes regulate pH_c by expelling protons through a H^+–adenosine triphosphatase and an amiloride-sensitive Na^+/H^+ antiporter (*11*). Physical localization or differential regulation of these pumps could account for localized proton efflux and the observed acidification of the medium surrounding the base of the rhizoid cell (*8*). Other factors may contribute to proton consumption or expulsion at the rhizoid base. Chloroplasts, for example, cause the surrounding cytoplasm to become more alkaline (*13*), and although their distribution has not been determined, chloroplast localization in the rhizoid base could contribute to regional cytoplasmic alkalinity.

We investigated the relation between the cytosolic pH gradient and rhizoid growth by manipulating pH_c with a membrane-permeant weak acid, propionic acid (PA) (*13*). The pH of artificial seawater (ASW) was lowered from 8.2 to 7.0 to increase the concentration of undissociated, membrane-permeant PA. Embryos developed normally in ASW at pH 7.0 and generated a cytosolic pH gradient of normal magnitude and orientation (Fig. 3A and Table 1). Addition of PA eliminated the cytosolic pH gradient and clamped pH_c to 7.4 to 7.5 (500 μM PA) or 6.8 to 6.9 (10 mM PA) (Fig. 3B). These PA treatments reduced the rhizoid elongation rate nearly fourfold (Fig. 3C and Table 1). Treated embryos completed multiple rounds of division on a normal time course. Because addition of PA altered pH_c in less than 30 s, dissipation of the gradient almost certainly preceded the cessation of tip elongation.

La^{3+}, a Ca^{2+} channel blocker that inhibits rhizoid growth (*14*), was used to further probe the relation between the pH

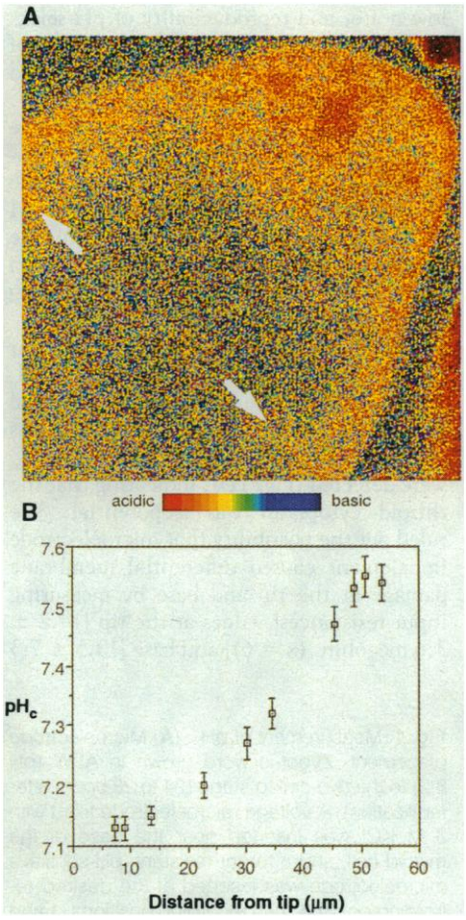

Fig. 2. Confocal ratio imaging of pH_c. Fluorescent images of a rhizoid cell (n = 9) injected with dextran-conjugated SNARF 1 (Molecular Probes, Eugene, Oregon) were collected at 580 ± 15 nm and 630 ± 10 nm. We divided the images using software provided with the Bio-Rad MRC 600 confocal microscope. The pH_c values in the unfiltered ratio image (**A**) were obtained from a standard curve made with buffered artificial cytoplasms. Mean pH_c values in a 20 by 20 pixel area were calculated at various positions along the rhizoid axis (**B**). Arrows indicate division plane. Bars are standard errors.

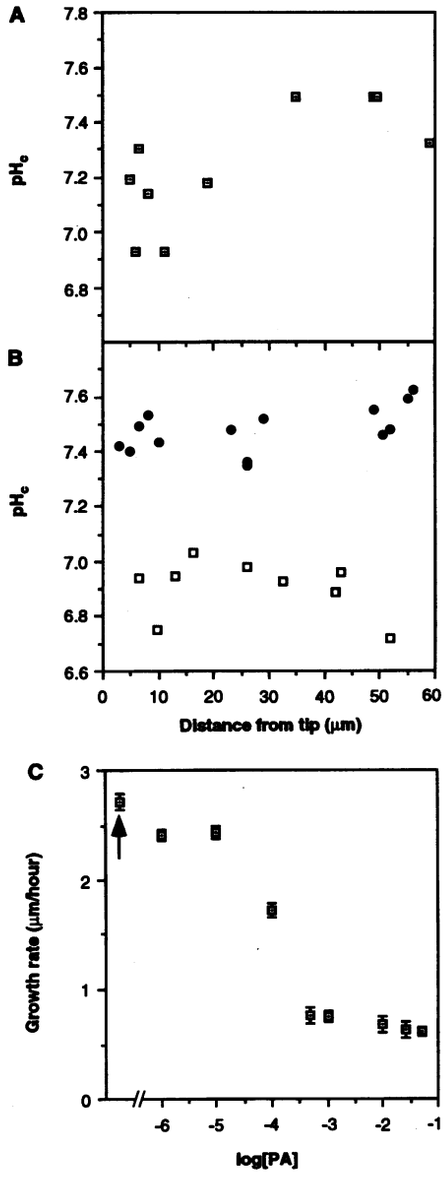

Fig. 3. Effect of PA treatment. Cells were rinsed once and incubated in ASW (pH 7.0) (**A**) or in ASW (pH 7.0) containing PA (**B**). PA was added at a total concentration of 500 μM (●) or 10 mM (□), and the concentration of undissociated, membrane-permeant PA was 3.7 μM or 74 μM, respectively. The pH_c measurements were made 20 to 120 min later. (**C**) Rhizoid growth rate. The axial length of 24-hour-old embryos was measured, ASW (pH 7.0) containing PA [0 (arrow) to 50 mM] was added, and axial lengths were measured again 24 hours later. Growth rates were calculated from the difference in the mean embryonic lengths at the two times. Bars represent standard deviations.

Table 1. Summary of pH_c measurements and rhizoid growth rates. The basal medium was ASW. The pH_c measurements for each treatment were pooled into apical (<15 μm from the tip) and basal (>40 μm from the tip) groups. Rhizoid growth rates were calculated as described in Fig. 3. $LaCl_3$ was added directly to ASW from a 1 M stock solution, and pH measurements were made 20 to 120 min later.

Treatment	Apical pH (n)	Basal pH (n)	P	Growth rate (μm/hour)
pH 8.2	7.15 ± 0.05(4)	7.45 ± 0.05(6)	<0.01	2.60 ± 0.07
pH 8.2 + 5 mM La^{3+}	7.34 ± 0.18(6)	7.34 ± 0.11(3)	0.98	0.02 ± 0.11
pH 7.0	7.10 ± 0.16(5)	7.43 ± 0.10(3)	0.02	2.71 ± 0.07
pH 7.0 + 500 μM PA	7.45 ± 0.05(5)	7.54 ± 0.07(5)	0.06	0.76 ± 0.07
pH 7.0 + 10 mM PA	6.88 ± 0.11(3)	6.86 ± 0.12(3)	0.82	0.68 ± 0.07

gradient and rhizoid elongation. Addition of 5 mM $LaCl_3$ dissipated the cytosolic pH gradient and stopped growth without significantly altering the average value of pH_c (Table 1). When all treatments were considered together, the magnitude of the cytosolic pH gradient correlated well with the rate of tip growth (Fig. 4).

Although we cannot be certain whether the cytosolic pH gradient is a cause or consequence of growth, the fact that protons serve as second messengers in all eukaryotic cells raises the possibility that the pH gradient regulates regional differentiation. Cytoplasmic proton activities are typically 40 to 60 nM in resting cells, similar to, or perhaps slightly lower than, the activity of cytosolic Ca^{2+}. Because of the low resting activity, a subtle change in H^+ metabolism or transport can alter pH_c and thereby trigger a cellular response. A number of morphogenetic processes, including egg activation, conidiation, germ tube formation, and cell differentiation are regulated in this fashion (*5*, *15*), and in most instances a moderate change in pH_c of a few tenths of a unit is sufficient for induction. These temporal pH_c triggers are similar in magnitude to the spatial pH_c gradient in *Pelvetia*, making it likely that regional rhizoid physiology is influenced by the gradient.

A number of cytoplasmic structures and processes are potential targets for differential regulation by the pH gradient; these include enzyme activity, membrane trafficking, exocytosis, and cytoskeletal stability (*16*). F-actin is localized in the cortical cytoplasm at the growing tip (*17*), the most acidic zone, whereas microtubules emanate from the perinuclear region (*18*), where pH_c is most alkaline. Microtubules radiate toward the apex, but are rare in the apical cortex. This general pattern of cytoarchitecture is common in tip-growing cells (*19*) and may be determined, at least in part, by the cytosolic pH gradient. Acidic conditions stabilize both plant and animal actin filaments (*20*), whereas microtubule assembly and stability are increased at alkaline pH (*21*).

Growing rhizoids also generate cytosolic and membrane-associated Ca^{2+} gradients that superimpose on the pH gradient (*2*, *14*). La^{3+} reduces cytosolic Ca^{2+} concentrations in the rhizoid (*14*) and eliminates the pH gradient, indicating that the pH gradient is dependent on Ca^{2+} homeostasis. Although the relation between the two gradients is unclear, it seems likely that they operate coordinately in regulating tip growth. The cytoskeleton is a plausible candidate for coordinate regulation because F-actin and microtubules are affected by both pH_c and cytosolic Ca^{2+} (*20*, *21*).

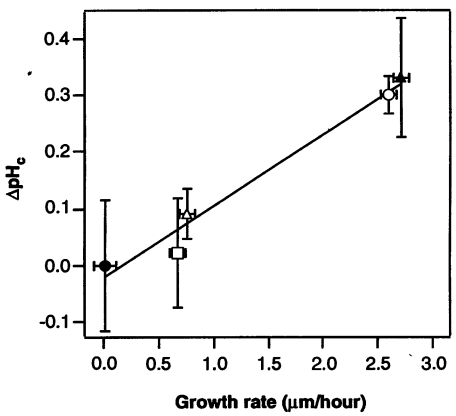

Fig. 4. Relation between the magnitude of the pH_c gradient (ΔpH_c) and rhizoid growth rate. We calculated ΔpH_c by subtracting the mean apical pH_c from the mean basal pH_c shown in Table 1. Correlation coefficient (r^2) = 0.97. ○, ASW pH 8.2; ▲, ASW pH 7.0; ●, ASW containing 5 mM $LaCl_3$; △, ASW containing 500 μM PA; and □, ASW containing 10 mM PA.

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Cell Fate Determination by the Cell Wall in Early *Fucus* Development

Frederic Berger, Alison Taylor, Colin Brownlee*

In multicellular plants, development starts with an asymmetric division of the zygote into two differentiated cells. The nature and distribution of fate-determining factors operating during embryogenesis remain largely obscure. Laser microsurgery was used here to dissect two-celled embryos of the alga *Fucus spiralis*. Removal of protoplasts from the cell wall induced dedifferentiation. However, isolated cells within the walls followed their restricted fate. Moreover, contact of one cell type with the isolated cell wall of the other cell type caused its fate to be switched. The cell wall thus appears to maintain the differentiated state and to direct cell fate in plant development.

Early development of *Fucus spiralis* follows a similar pattern to zygotic embryogenesis in higher plants (*1*), the first asymmetric cell division giving rise to a rhizoid cell that develops into the basal root–like tissue (holdfast) and an apical thallus cell that produces the vegetative and reproductive shoot. Polarization processes taking place before the first cell division have been extensively studied (*2*). The use of enzymatically produced wall-free protoplasts has shown that the cell wall is required for fixation of the polar axis (*3*). It is assumed that bridges involving a vitronectin-like protein connect the cell wall to the plasma membrane (*4*). However, mechanisms in-

F. Berger, Marine Biological Association, The Laboratory, Citadel Hill, Plymouth, PL1 2PB United Kingdom, and Ecole Normale Supérieure de Lyon, Laboratoire de Biologie Cellulaire et Moléculaire, F-69364, Lyon Cedex 07 France.

A. Taylor and C. Brownlee, Marine Biological Association, The Laboratory, Citadel Hill, Plymouth, PL1 2PB United Kingdom.

*To whom correspondence should be addressed.