Ionic currents and ion fluxes in *Neurospora crassa* hyphae

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Abstract

Voltage dependence of ionic currents and ion fluxes in a walled, turgor-regulating cell were measured in *Neurospora crassa*. The hyphal morphology of the model organism *Neurospora* simplifies cable analysis of ionic currents to determine current density for quantitative comparisons with ion fluxes. The ion fluxes were measured directly and non-invasively with self-referencing ion-selective microelectrodes. Four ions (H⁺, Ca²⁺, K⁺, and Cl⁻) were examined. H⁺ net uptake and Ca²⁺ net release were small (10.2 nmol m⁻² s⁻¹ and 1.1 nmol m⁻² s⁻¹, respectively) and voltage independent. K⁺ and Cl⁻ fluxes were larger and voltage dependent. Maximal K⁺ net release (~1440 nmol m⁻² s⁻¹) was observed at positive voltages (+15 mV), while maximal Cl⁻ net release (~905 nmol m⁻² s⁻¹) was observed at negative voltage (~210 mV). A possible function of the net outward K⁺ and Cl⁻ fluxes is regulation of the plasma membrane potential. Total ion fluxes were 37–58% of the total ionic current density (about ±244 mA m⁻², equivalent to ±2500 nmol m⁻² s⁻¹, at 0 mV and ±200 mV) so other ions must contribute significantly to the ionic currents.

Key words: Current clamp, filamentous fungi, K⁺, Cl⁻, H⁺, and Ca²⁺ fluxes, ion-selective microelectrodes, voltage clamp, walled turgid cells.

Introduction

Ion transport at the plasma membrane is crucial to cellular life. Nutrient uptake, osmotic regulation, and signalling can require the movement of ions either into or out of the cell. Much of this transport involves net charge movement and is thus electrogenic in nature, contributing to the electrical properties of the cell. Although the relationship between the ionic currents at the plasma membrane and the corresponding ionic fluxes can be inferred using radioactive tracers, direct, simultaneous measurements of ionic currents and fluxes can now be performed non-invasively using ion-selective microelectrodes. With this technique, the external diffusive gradient created by ion transport across the plasma membrane is sampled with the ion-selective microelectrode at two positions, one near to and one far from the cell; the ion flux can be calculated from the difference in ion concentration (Smith et al., 1999). Ion flux measurements can be performed during voltage clamp or other electrical manipulations of the cell. Babourina et al. (2001) and Tyerman et al. (2001) performed simultaneous measurements of the electrical properties of the cell and ion fluxes (with the ‘MIFE’ technique, www.mife.com) (Newman, 2001), by voltage clamping either root hairs (Lew, 1991) or protoplasts (with whole-cell patch-clamp), respectively. Simultaneous patch-clamp and ion flux measurements have revealed the contribution of ion fluxes to known ion channel or transport activities in protoplasts (Fuster et al., 2004; Gilliham et al., 2006) and giant membrane patches (Kang et al., 2003; Kang and Hilgemann, 2003). The juxtaposition of the two techniques (patch-clamp and ion-selective microprobes) is powerful and integrative.

In plant and fungal organisms, the cells are typically walled, and normally under hydrostatic pressure (turgor). To patch-clamp the cell, the wall must first be removed (either enzymatically or with laser ablation). To avoid cell bursting, the cells must be plasmolysed. This is a physiologically abnormal state since cellular growth and division require a wall. Furthermore, plant and fungal cells modulate ion transport to regulate turgor (Shabala and Lew, 2002; Lew et al., 2004, 2006), so transport properties of the protoplast will be different from those of the intact turgid cell. Even when an intracellular voltage clamp on intact cells of roots is used in conjunction with ion flux measurements (Babourina et al., 2001), ionic currents between cells via plasmodesmata—as measured by significant electrical coupling between adjacent root...
cells (Lew, 1994, 1996)—means that current density (A m⁻²) cannot be quantified for comparisons with ion fluxes. To address these issues and problems, the fungal organism Neurospora crassa offers a number of advantages. The hyphal morphology is simpler than the multicellular three-dimensional architecture of plant roots, so cable analysis (Rall, 1977) can be used to determine current density. The hyphae are amenable to ion flux measurements with the ion-selective probe (Lew, 1999). It shares many transport properties with higher plants. Like higher plant cells, the dominant plasma membrane transport is the H⁺-ATPase. Out of a total of 64 transporter families identified in the genomes of either Arabidopsis thaliana or N. crassa, 39 are found in both (http://www.membranetransport.org). It regulates turgor (Lew et al., 2004) similarly to the higher plant model organism A. thaliana (Shabala and Lew, 2002). Therefore, N. crassa is an appropriate and accessible model for a walled, turgid cell, with genomic and physiological similarities to other organisms that allow it to be useful as a model and guide for transport processes in both plant and fungal cells.

The objective of the present study was to characterize the electrical properties of the fungal hyphal cell and identify the ions responsible for voltage-dependent ionic currents in N. crassa as a model for walled, turgor-regulating organisms. The results are direct evidence that K⁺ and Cl⁻ fluxes are central to regulation of the membrane potential of the intact turgid cell, but other ions also play a significant role.

Materials and methods

Strains
Stock cultures of wild-type N. crassa (strain 74-OR23-1A, FGSC No. 987) were obtained from the Fungal Genetics Stock Center (School of Biological Sciences, University of Missouri, Kansas City, MO, USA) (McCulskey, 2003), and maintained on Vogel’s medium [plus 1.5% (w/v) sucrose and 2.0% (w/v) agar] (VM) (Vogel, 1956). The major ions in VM (in mM) are: K⁺ (36.7), Pᵢ (36.7), Na⁺ (25.5), NH₄⁺ (25), NO₃⁻ (25), citrate (8.5), Cl⁻ (1.36), Mg²⁺ (0.81), SO₄²⁻ (0.81), and Ca²⁺ (0.68); pH is 5.8.

Culture preparation for experiments
Cultures were grown at 28 °C (or room temperature, 21–24 °C) overnight by transferring conidia onto strips (2.5×3 cm) of dialysis tubing that overlay the VM in Petri dishes. The dialysis tubing was cut with a razor blade to a size of about 1×3 cm, which included the growing edge of the colony, placed inside the cover of a 30 mm Petri dish, and immobilized on the bottom with masking tape or double-sided sticky tape. The culture was flooded with 3 ml of the growth medium of the selected ion was measured as near as possible

mycelia were incubated in the modified (unbuffered) BS, the pH was 5.5 (±0.08) and stable throughout the 30–45 min duration of the experiments, thus buffering effects on H⁺ flux measurements were very small. Growth of hyphae at the colony edge normally resumed within 15 min in either BS or modified BS, indicating that the lower ionic strength of the modified BS had no adverse effect on hyphal physiology.

For electrophysiology and flux measurements, large trunk hyphae (10–20 µm diameter) in situ about 0.5 cm behind the colony edge were used.

Electrical measurement of hyphal cable properties
The hyphae were impaled with a double barrel micropipette (Lew, 2006) and a single barrel micropipette some distance away from the first impalement site (Fig. 1A). The micropipettes were pulled from borosilicate glass capillaries (OD 1.0 mm, ID 0.58 mm, with filament; Friedrich and Dimmock Inc., Millville, NJ, USA) on a Sutter P-30 (Sutter Instruments Co., Novato, CA, USA) puller modified for pulling double barrel micropipettes. Both micropipettes were filled with 3 M KCl. The circuit was completed with a salt bridge containing 3 M KCl in 2% (w/v) agar and an Ag/AgCl electrode. The voltage clamping apparatus has been described in detail elsewhere (Lew, 1996). Briefly, the double barrel micro-electrodes were connected to IE-251 electrometers (input impedance 10¹¹ Ω; Warner Instruments, Hamden, CT, USA). A data acquisition board (Scientific Solutions, Solon, OH, USA) was used to control an operational amplifier configured for voltage clamp. During the last 10 ms of the voltageclamp, the clamped voltage (Vₓ) was sampled (to ensure clamping fidelity), as was the clamping current. The single barrel microelectrode was connected to an IE-201 electrometer (input impedance 10¹⁵ Ω; Warner Instruments) and the hyphal voltage (Vᵧ) (at a distance, d, further along the hypha) measured at the same time as Vₓ. Voltage attenuation at the distance d was calculated from the slope of the relationship (always linear) of Vᵧ versus Vₓ.

Voltage dependence of ion flux
To measure the effect of hyphal voltage on ion fluxes, current clamping was used to modify the cell potential. The hypha was impaled with two single barrel micropipettes filled with 3 M KCl. Cytoplasmic flow was often observed after the impalements (Fig. 2A, B). One micropipette injected current into the hypha (I₁), and the other measured the cell potential (V₁) (Fig. 2B, C). The micropipettes were pulled from borosilicate glass capillaries (OD 1.2 mm, ID 0.68 mm, with filament) (catalogue no. 1B120F-4, Sigma-Aldrich Corp, St Louis, MO, USA) were: H⁺ (Hydrogen Ionophore II–Cocktail A, catalogue number 95297), Ca²⁺ (Calcium Ionophore I–Cocktail A, catalogue number 21048), K⁺ (Potassium Ionophore I–Cocktail B, catalogue number 60398), and Cl⁻ (Chloride Ionophore I–Cocktail A, catalogue number 24902). The concentration of the selected ion was measured as near as possible...
to the hyphal wall and 20 μm away at a frequency of 0.3 Hz (Fig. 2). The ion-selective electrode will also have a small electrical potential, which should be constant in the extracellular medium, and thus cancel out when differences in ion concentration are measured. Organic substance, which affected the voltage offset, but not the slope of voltage versus [Cl–]. This offset was corrected by normalizing to the expected concentration of 0.2 mM [Cl–] for flux calculations. Concerns have been raised that Cl– selectivity may be an estimator of population variance) unless stated otherwise.

**Statistical analysis**

Statistics are shown as mean ± SD (sample size) (SD was used as an estimator of population variance) unless stated otherwise. Independent two-tail t-tests were performed in either SYSTAT (Systat, Inc.) or Excel (Microsoft).

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**Fig. 1.** Cable properties of *Neurospora crassa* hyphae. (A) Images of hyphae impaled with double barrel and single barrel micropipettes to measure voltage attenuation at hyphal lengths of about 0, 125, or 450 μm (left, centre, and right panel, respectively). Arrows mark the locations of septa. Scales are as marked. (B) Time dependence of voltage clamp. Voltage clamp durations were varied from 25 ms to 400 ms to determine the duration that maximized voltage clamp fidelity. Voltage attenuation (V0/Vd) where V0 is the voltage measured at the voltage clamping microtip and Vd the voltage measured at distance d from the voltage clamping site) was lowest with a duration of 50 ms, independent of the distance (7.5 μm, circles; 120 μm, squares; or 460 μm, triangles, as shown) between the double barrel micropipette used to voltage clamp, and the single micropipette used to measure voltage. Both individual experiments (lines) and means ± SD (symbols) are shown. (C) Voltage attenuation as a function of distance along the hyphae revealed the cable properties of the hyphae. The curve is a best fit to an exponential function V0/Vd = e^(-λd), where λ, the length constant, was 407 μm.

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**Fig. 2.** Example of dual impalements for current clamp and concurrent ion flux measurements. A (0 min) and B (1.4 min) show hyphal flow immediately after impalements. The diagonal arrows point to a vacuole moving towards the colony edge. B and C show the experimental protocol. The current (usually ±2.5, ±5, and ±10 nA) was injected through the micropipette marked I1. Voltage was monitored with the micropipette marked V1. The ion-selective probe continuously measured ion fluxes during the current clamp. The ion concentrations (c2 and c1) were measured at the distances marked r1 and r2, and used to calculate the ion fluxes.

To avoid changes in the ion-selective probe output (μV) in response to the current injections (nA) into the hypha, two reference electrodes were used (both filled with 3 M NaCl in 2% agar), one connected to the Duo-773 electrometer, and the other connected to the ion-selective probe amplifier.

Ion fluxes were calculated from the ion concentration differences taking into account the cylindrical geometry of the hyphae: \( J = \frac{\Delta N}{A \cdot t} = \frac{(c_2 - c_1)}{(r_2 - r_1)} \frac{D}{R} \) where \( D \) is the diffusion coefficient (H+, \( 9.31 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \); Ca2+, \( 0.4 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \); K+, \( 1.96 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \); Cl–, \( 2.03 \times 10^{-5} \text{ cm}^2 \text{ s}^{-1} \)), \( r \) is the hyphal radius, \( c_2 \) and \( c_1 \) are the concentrations at the two excursion points, and \( r_2 \) and \( r_1 \) are the distances from the hyphal centre of the two excursion points (Henriksen et al., 1992). The Cl−-selective cocktail registered higher than expected [Cl−] in the modified BS. Additions of [Cl−] to the solution caused the predicted voltage change, indicating that there was interference, probably caused by some organic substance, which affected the voltage offset, but not the slope of voltage versus [Cl−]. This offset was corrected by normalizing to the expected concentration of 0.2 mM [Cl−] for flux calculations. Concerns have been raised that Cl− selectivity may be low compared with anionic bases (e.g. the buffer MES), so that the Cl− probe is responding indirectly to H+ fluxes (Messerli et al., 2006). Measurements of net H+ and Cl− fluxes at growing tips in *Neurospora crassa* hyphae. (A) Images of hyphae impaled with double barrel and single barrel micropipettes to measure voltage attenuation at hyphal lengths of about 0, 125, or 450 μm (left, centre, and right panel, respectively). Arrows mark the locations of septa. Scales are as marked. (B) Time dependence of voltage clamp. Voltage clamp durations were varied from 25 ms to 400 ms to determine the duration that maximized voltage clamp fidelity. Voltage attenuation (V0/Vd) where V0 is the voltage measured at the voltage clamping microtip and Vd the voltage measured at distance d from the voltage clamping site) was lowest with a duration of 50 ms, independent of the distance (7.5 μm, circles; 120 μm, squares; or 460 μm, triangles, as shown) between the double barrel micropipette used to voltage clamp, and the second micropipette used to measure voltage. Both individual experiments (lines) and means ± SD (symbols) are shown. (C) Voltage attenuation as a function of distance along the hyphae revealed the cable properties of the hyphae. The curve is a best fit to an exponential function V0/Vd = e^(-λd), where λ, the length constant, was 407 μm.
Results

To determine the current density \( (A \, m^{-2}) \) of the hypha at various voltages, it was necessary to assess the extent to which voltage was affected by current injection as a function of distance along the hypha. From this characterization of the cable properties of the hypha, the current density could be compared with the ion fluxes measured for \( H^+ \), \( Ca^{2+} \), \( K^+ \), and \( Cl^- \).

Hyphal cable properties

To determine the extent of current leakage out of the cable-like hypha, multiple impalements were performed. Voltage clamping was performed using a double barrel micropipette; voltage attenuation was measured with a single barrel micropipette impaled about 8, 120, or 460 \( \mu m \) away from the double barrel impalement site on the same hypha (Fig. 1A). In preliminary experiments, the duration of the voltage clamp was varied from 25 ms to 400 ms to determine the optimal duration for voltage clamp fidelity. Maximal voltage response was observed with a clamp duration of 50 ms, independent of the distance (Fig. 1B). Input conductance at the site of voltage clamping was 0.49\( \pm 0.30 \, \mu S \) \((n=55)\) and was unaffected by the duration of the clamp (correlation coefficient, \( r^2=0.01 \)). The effect of distance on voltage attenuation \( (V_d/V_0) \) was fit with an exponential function \( V_d/V_0=\exp((x-\lambda)/k \mu m) \), the length constant, \( \lambda \) was 407 \( \mu m \) (Rall, 1977) (Fig. 1C). Septa along the hypha offered no significant impediment to current flow along the hyphal cable. For hyphae 450–500 \( \mu m \) long having either three \((n=4)\) or four \((n=3)\) septa between the two microelectrode impalement sites (the data are shown in Fig. 1C), voltage attenuation was not significantly different \((P=0.365)\). This is not unexpected, since mass flow through septa (Lew, 2005) was commonly observed during experimental measurements, implying minimal impedance to the flow of ionic currents. In addition to unimpeded cytoplasm flow, the septal length is relatively short \((\sim 1–2 \, \mu m)\) compared with the overall length of hypha, minimizing the effect of the septa on the cable resistance of the hypha.

Based upon the length constant of 407 \( \mu m \), for ion flux measurements, if the ion-selective probe is in close proximity \((within 20–60 \, \mu m)\) to the current-injecting micropipette, voltage should be relatively unattenuated.

Current clamp and ion fluxes

To measure net ion fluxes, the ion-selective probe was positioned near the voltage-monitoring micropipette and within 40–60 \( \mu m \) of the current-injecting micropipette (Fig. 2). After initial measurements of micropipette resistance, the hypha was impaled; first with the voltage-monitoring micropipette, then with the current-injecting micropipette. The voltages of both micropipettes were monitored when current injections were not occurring to ensure that the values were similar, indicating that the hypha was impaled by both micropipettes. Furthermore, if current injection did not cause a change in the voltage of the voltage-monitoring micropipette, re-impalement was attempted (if unsuccessful, the experiment was aborted). After successful impalements, the ion-selective microelectrode was positioned near the voltage-monitoring micropipette (Fig. 2) and net ion flux measurements commenced.

Net ion fluxes were measured continuously during current clamp (Fig. 3). The usual current injection protocol was \(-2.5 \, nA, +2.5 \, nA, 0 \, nA, -5 \, nA, +5 \, nA, 0 \, nA, \) etc. When current injections were started, it was not uncommon to see transient changes in the \( \mu V \) reading from the ion-selective microelectrode, which were discarded from further analysis. As a control, at the end of the experiment, the ion-selective electrode was moved away from the hypha \((\sim 60 \, \mu m)\) and a set of final current injections performed (Fig. 3), to confirm that the ion-selective electrodes were reporting net ionic fluxes rather than artefactual signals, which could be caused by slight changes in the voltage at the reference electrodes.

The current clamp will introduce either \( K^+ \) or \( Cl^- \) into the hypha. Currents of \( \pm 10 \, nA \) correspond to a total coulombic injection of \( 1.6 \times 10^{-6} \) coulombs for a typical 160 s injection \((1.7 \times 10^{-11} \, mol \) after conversion with the Faraday constant). The maximal concentration can be estimated at about 51 mM \((assuming K^+ or Cl^- contributes half of the total coulombic injection) based on a hyphal volume using the value for \( 2 \times \lambda \) (the length

![Fig. 3](https://academic.oup.com/jxb/article-abstract/58/12/3475/637577)
constant based on hyphal cable properties, \( \lambda \), multiplied by 2 to account for ion flow in both directions along the hypha as the effective hyphal length and the average radius of the hyphae (8.0±1.1 \( \mu \)m, \( n=13 \); \( \pi \times r^2 \times \lambda \times 2 = 1.6 \times 10^{-10} \) l. This is a significant increase in ion concentration in the hypha. However, the hyphal membrane potentials when no current was being injected exhibited no trend, either depolarizing or hyperpolarizing, during the course of the experiment, so the electrical properties of the hypha appeared to be unaffected by the prolonged current injections (Fig. 4). Furthermore, mass flow (i.e. cytoplasmic movement along the hypha) towards the growing colony edge was often observed during the current clamping experiments (whether or not current was being injected) (Fig. 2A, B). Mass flow is caused by small pressure differences along the hypha, effecting non-turbulent cytoplasm movement due to the low Reynolds number of the hyphal tube (Lew, 2005), and is indirect proof of continued cell viability during the experiments. Mass flow, in addition to diffusion and the intrahyphal ion current, would act to disperse the injected ions away from the site of impalement during current clamp.

The current–voltage relationship and voltage dependence of ion fluxes are shown in Fig. 5. K\(^+\) release (Fig. 5B) and Cl\(^-\) release (shown as the flux of electric charge for comparison with the current–voltage relationship) (Fig. 5C) increased at positive and negative voltages, respectively. For all voltages, the net fluxes were (K\(^+\)) (outward) 195±683 (\( n=47 \)) and (Cl\(^-\)) (outward) 530±681 (\( n=32 \)) nmol m\(^{-2}\) s\(^{-1}\). By comparison, H\(^+\) and Ca\(^{2+}\) fluxes (Fig. 5D) were much smaller in magnitude [inward at 10.2±16.7 (\( n=19 \)) and outward at 1.1±1.8 (\( n=6 \)) nmol m\(^{-2}\) s\(^{-1}\), respectively], and were voltage independent.

The current densities were calculated from the input currents based upon the length constant (\( \lambda \)) (Rall, 1977) obtained from measurements of the cable properties of the hyphae. The cable current (\( i_m \), in units of A m\(^{-2}\)) was calculated from the input current (\( I \)):

\[
i_m = \frac{I}{(2 \times \lambda)}
\]

where the factor 2 accounts for current flow in both directions along the hypha from the site of current injection. Current density (\( i_m \), in units of A m\(^{-2}\)) was calculated based on measurements of the hyphal diameter (\( d \), 16.0±2.2 \( \mu \)m, \( n=13 \)):

\[
i_m = \frac{I_m}{(\pi \times d)}
\]

Input currents of ±10 nA generate ±244 mA m\(^{-2}\), equivalent to monovalent ionic fluxes of ±2500 nmol m\(^{-2}\) s\(^{-1}\). This is about 1.7- and 2.8-fold higher than the net outward fluxes measured for K\(^+\) and Cl\(^-\) fluxes.
(1440 nmol m$^{-2}$ s$^{-1}$) and Cl$^-$ (905 nmol m$^{-2}$ s$^{-1}$) at positive (14±14 mV) and negative voltages (−210±31 mV), respectively.

**Discussion**

While patch-clamp has been a powerful technique for characterizing ion transport, for plant, fungal, and algal cells, the protoplasts required for patch-clamp measurements of walled cells are far removed from the normal physiological state of a walled turgid cell. In addition, whole-cell measurements with the patch-clamp technique will modify the cytoplasm drastically due to mixing of the patch pipette contents with the cytoplasm that will modify concentrations of small metabolites and signalling molecules. Direct measurements of ion fluxes on walled cells, in tandem with an intracellular voltage clamp, can sample ion transport in fungal and plant cells in a dynamic living state. *Neurospora crassa* is an ideal model organism for such measurements, primarily because of the relatively simple cable geometry of the hyphal network. It exhibits ion transport responses similar to those of higher plants (Shabala and Lew, 2002) during turgor regulation after hyperosmotic treatment (Lew et al., 2004, 2006) and shares many transporter families with *A. thaliana*. Most notable is the central role of the plasma membrane H$^+$-ATPase in both plants and fungi.

Using *N. crassa* hyphae, it was possible to identify the dominant voltage-dependent ion fluxes at the plasma membrane of a walled, turgid cell. The viability of the hyphae was assured since tip growth continued at the colony edge, and by the common observation of mass flow though the hyphal ‘tubes’ supplying cytoplasm to the colony edge (Lew, 2005). To determine the voltage dependence of ion flux, the measurements had to be carried out under conditions in which current spread through the cell was well understood. The cable-like morphology of the hypha made them an excellent system for determining current density, allowing direct comparisons between the hyphal ionic currents and ion fluxes. The current densities (∓244 mA m$^{-2}$ for input currents of ∓10 nA) are similar to those reported by Gradmann et al. (1978) (in the range of 100–300 mA m$^{-2}$ for voltages positive and negative to the resting potential of *Neurospora*). The contributions of H$^+$, Ca$^{2+}$, K$^+$, and Cl$^-$ fluxes to the inward and outward currents were examined. H$^+$ and Ca$^{2+}$ net fluxes were very small. The net H$^+$ uptake is presumably due to a balance between H$^+$ release by the H$^+$-ATPase and uptake via H$^+$ co-transporters. The small net Ca$^{2+}$ release is presumably related to regulation of cytoplasmic Ca$^{2+}$ concentrations. Instead, much of the ionic current at negative and positive voltages (relative to the typical plasma membrane potential) was carried by Cl$^-$ and K$^+$ net release, respectively. The K$^+$ net efflux is probably mediated by an outward rectifying K$^+$ channel (NcTOKA) that has been characterized with the patch-clamp technique after heterologous expression in yeast (Roberts, 2003). The total net ion flux is smaller than the net ion flux expected from the current density. Other ions must contribute to the ionic current. Likely possibilities are fluxes of Na$^+$, NH$_4^+$, P$_i$, NO$_3^-$, and organic acids, since the inorganic ions Na$^+$, NH$_4^+$, P$_i$, and NO$_3^-$ are supplied at high concentration in VM, and organic acids would be produced during respiration.

In summary, the voltage dependence of ionic currents and ion fluxes has been determined directly and quantitatively in a walled, turgid cell. At voltages positive and negative to the resting potential, the major contributors to ion fluxes are outward K$^+$ (58%) and Cl$^-$ (37%) net fluxes. Net H$^+$ and Ca$^{2+}$ fluxes are very small in comparison. The voltage dependence of K$^+$ and Cl$^-$ outward net fluxes suggests a primary role in ‘clamping’ the membrane potential. Other ions must contribute significantly to the ionic currents at voltages positive and negative to the normal resting potential of the cell.

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