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METHODS & TECHNIQUES

Localization of K^+, H^+, Na^+ and Ca^{2+} fluxes to the excretory pore in *Caenorhabditis elegans*: application of scanning ion-selective microelectrodes

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ABSTRACT

Although *Caenorhabditis elegans* is commonly used as a model organism for studies of cell biology, development and physiology, the small size of the worm has impeded measurements of ion transport by the excretory cell and hypodermis. Here, we use the scanning ion-selective microelectrode technique to measure efflux and influx of K^+, H^+, Na^+ and Ca^{2+} in intact worms. Transport of ions into, or out of, immobilized worms produces small gradients in ion concentration in the unstirred layer near the surface of the worm. These gradients are readily detectable with ion-selective microelectrodes and the corresponding ion fluxes can be estimated using the Fick equation. Our data show that effluxes of K^+, H^+, Na^+ and Ca^{2+} are localized to the region of the excretory pore, consistent with release of these ions from the excretory cell, and that effluxes increase after experimental preloading with Na^+, K^+ or Ca^{2+}. In addition, the hypodermis is a site of Na^+ influx.

KEY WORDS: Nematode, Excretory cell, Hypodermis, Ion-selective microelectrodes

INTRODUCTION

The importance of the nematode *Caenorhabditis elegans* Maupas 1900 as a model organism for studies of the physiological and molecular mechanisms of salt and water homeostasis has been highlighted in a recent review (Choe, 2013). As with other areas of *C. elegans* research, studies of excretory function are aided by the ease of culture, the availability of a fully-sequenced genome, low-cost access to mutant strains, the invariant lineage of somatic cells and the relative ease with which expression of specific genes can be silenced using RNA interference techniques. The excretory system of *C. elegans* consists of just three cells: an excretory cell, duct cell and pore cell. The large, H-shaped excretory cell forms two canals which run along much of the length of the worm. Ultrastructural studies show that the output of the excretory cell passes through the duct cell to an excretory pore, which opens to the outside of the animal just anterior to the pharynx (Nelson et al., 1983). Laser ablation of any of the three cells of the excretory system results in worm swelling and eventual mortality, implicating these cells in water homeostasis (Nelson and Riddle, 1984). The excretory cell is richly endowed with ion channels and transporters, including Cl^- channels, Na^+/H^+ exchangers, the vacuolar-type H^+-ATPase and aquaporins (Choe, 2013). However, the drawbacks to the use of *C. elegans* in studies of ion excretion and osmoregulation are the thick cuticle and small size of the worms, greatly complicating the collection and analysis of the excretory fluids (Choe, 2013). The transport functions of the excretory cell have thus been largely unexplored because of the technical challenges inherent in studies of such a small organism.

In this paper, we describe a new method for analysis of ion efflux and influx by intact worms. Transport of ions into or out of worms bathed in saline produces gradients in ion concentration in the unstirred layer adjacent to the surface of the worm. These gradients can be quantified using the scanning ion-selective electrode technique (SIET), whereby voltages are recorded from an ion-selective microelectrode moved between two points within the unstirred layer. This method provides a means for precise localization of the sites of ion uptake and release.

RESULTS AND DISCUSSION

Fig. 1 shows voltage differences recorded by ion-selective microelectrodes (K^+, H^+, Na^+ and Ca^{2+}) positioned near the surface of adult *C. elegans*. The length of each arrow in Fig. 1 indicates the voltage difference when the microelectrode was moved between a position within 3–5 µm of the surface of the worm and a second position 50 µm further away. Arrows pointing away from the worm indicate that the voltage was more positive at the inner limit, consistent with a higher cation concentration in the unstirred layer near the surface of the worm relative to the outer position.

For each of the four ions, a clear pattern of voltage gradients was apparent. The voltage difference was maximal on one side of the animal at a point just anterior to the junction of the pharynx and the intestine. The voltage differences indicated highly localized increases in the concentrations of K^+, H^+, Na^+ and Ca^{2+} at a site consistent with the opening of the excretory pore, just anterior to the pharynx. Plots such as those in Fig. 1 were used to measure the distances from the anterior tip of the worm to the site of maximal voltage difference and from the tip to the readily identifiable junction of the pharynx and the intestine. For 38 worms, the mean distance to the site of the maximal voltage difference recorded by SIET was 133.9±5.1 µm (mean ± s.e.m.) and the distance to the junction of the pharynx and the intestine was 161.2±4.2 µm. When the distance to the site of maximal voltage difference was expressed as a proportion of the distance to the junction of the pharynx and the intestine, the values for K^+ (0.86±0.03; N=11 worms), H^+ (0.81±0.03, N=7), Na^+ (0.83±0.05, N=13) and Ca^{2+} (0.80±0.03, N=7) were not significantly different. These results indicate that effluxes of all four cations peak at a single location on one side of the animal. There was no evidence of ion efflux from the mouth in any of the measurements, indicating that exposure to levamisole is not associated with leakage of the contents of the intestine or pharynx.

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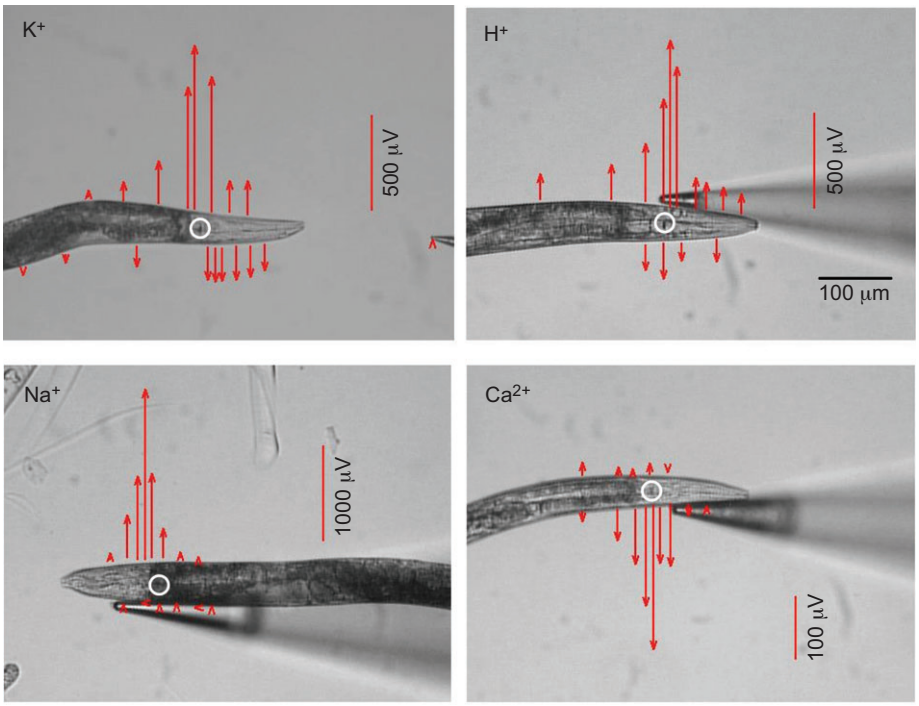

Fig. 1. Representative scans showing voltage differences recorded by SIET at locations near the anterior end of adult *Caenorhabditis elegans*. Bathing saline ion concentrations for K^+, H^+, Na^+ and Ca^{2+} measurements were 0.15 mmol l^{-1}, 10^{-7} mol l^{-1}, 0.15–3 mmol l^{-1} and 10^{-5} mol l^{-1}, respectively. The white circle in each panel indicates the lumen of the posterior bulb of the pharynx. In each case, the microelectrode was first positioned within 3–5 µm of the surface of the worm, at the site indicated by the base of the outwardly-directed red arrows. The microelectrode tip was then moved to a second position 50 µm further away. The voltage difference was calculated from the voltages at inner and outer limits of microelectrode excursion. The length of the arrow corresponds to the mean voltage difference of three replicate measurements at each site. Voltage scales are provided in the top right of each panel. Scale bar for all images is shown in H^+ panel.

Voltage gradients at each site were converted into concentration gradients as described in the Materials and methods and ion fluxes (pmol cm^{-2} s^{-1}) were then estimated from the measured voltage differences using the Fick equation. Plots of the fluxes for each ion as a function the distance anterior or posterior from the site of maximal ion flux are shown in Fig. 2. K^+ effluxes were restricted to a narrow zone of <50 µm anterior or posterior to the site of maximal flux. Although this pattern was apparent for K^+ fluxes for animals taken directly from the culture dishes, the maximal flux increased if worms were exposed to elevated K^+ levels (Fig. 2A; 89 mmol l^{-1}) for periods of 40−150 min before SIET measurements. The high K^+ conditions were produced by replacement of NaCl with KCl in M9 buffer. At distances of >100 µm posterior to the site of maximal efflux, K^+ fluxes declined to small effluxes or, in a few worms, a

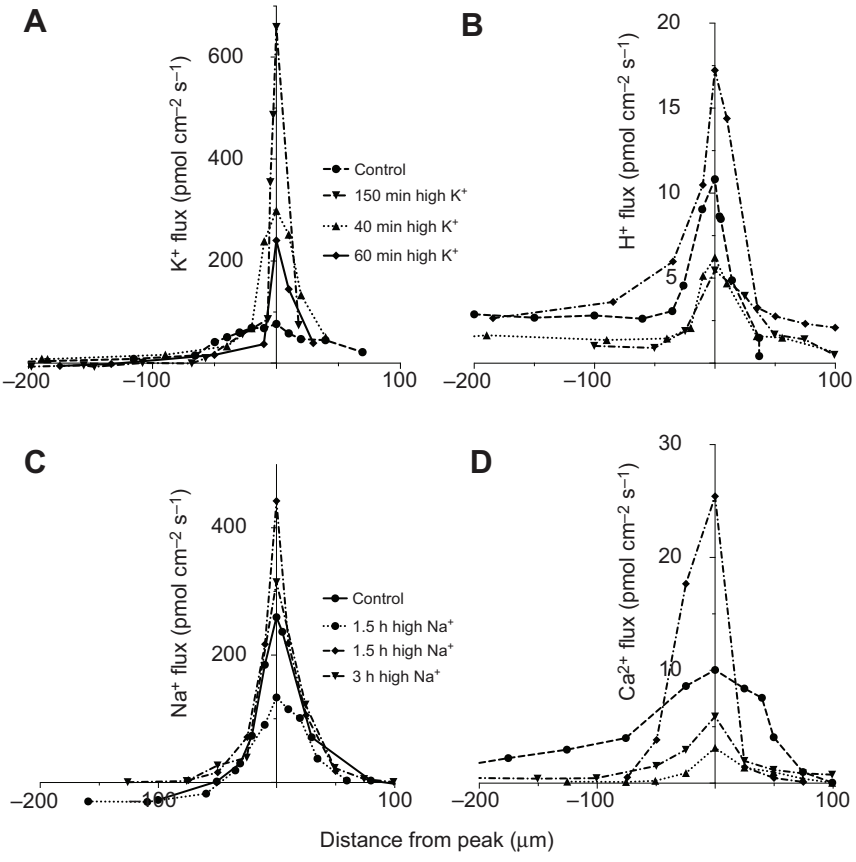

Fig. 2. Localization of ion fluxes in adult *Caenorhabditis elegans*. Fluxes (pmol cm^{-2} s^{-1}) were calculated from ion-selective microelectrode voltage differences at 7–15 locations along the anterior 250–300 µm of each worm. In each panel, data for a single worm are denoted by separate symbols and lines. Positive values along the axis correspond to microelectrode positions anterior to the site of maximal efflux and negative values correspond to positions posterior to the site of maximal efflux. Control worms were transferred directly from NGM plates to the saline used for SIET measurements. (A,C) High K^+ and high Na^+, worms were incubated for the indicated periods in K^+-rich or Na^+-rich saline, as described in the text, before transfer to the saline used for SIET measurements. (B) For H^+ flux measurements, all worms were transferred directly from NGM plates to saline used for SIET measurements. (D) For Ca^{2+}, all worms were incubated for 1−2 h in saline containing 150 mmol l^{-1} NaCl and 20 mmol l^{-1} $CaCl_2$ before transfer to the saline used for SIET measurements. Each line indicates a single worm.

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small influx; the mean flux was 1.82±1.32 pmol cm^{-2} s^{-1} (N=10). H^+ effluxes were restricted to a slightly wider zone, ~100 µm on either side of the site of maximal efflux (Fig. 2B). However, H^+ effluxes of 1–3 pmol cm^{-2} s^{-1} were measured at distances of 200 µm or more posterior to the site of maximal efflux.

Localized Na^+ fluxes were detected in less than half of the worms that were transferred directly from the culture medium (Fig. 2C). However, exposure of the worms to a high, but survivable (Choe and Strange, 2008), concentration of NaCl (400 mmol l^{-1}) for 1.5–3 h was associated with highly regionalized efflux, just anterior to the pharynx and consistent with the location of the excretory pore. However, there was also evidence of Na^+ influx at regions more than ~100 µm posterior to the site of maximal efflux, consistent with inward transport of Na^+ across the hypodermis at locations posterior to the excretory pore. The mean value of the influx was −20.4±2.7 pmol cm^{-2} s^{-1} (N=10 worms); values in worms taken directly from culture and those that had been preloaded with Na^+ by exposure to 400 mmol l^{-1} NaCl for 1.5–3 h did not differ and the results for both groups were therefore pooled. We also measured Na^+ uptake across the hypodermis in animals bathed in a moderately hard reconstituted water (MHRW) (Khanna et al., 1997), which approximates the composition of soil water and contains 2.1 mmol l^{-1} Na^+. Measurements of worms taken directly from the culture medium indicated that Ca^{2+} fluxes were small and were not localized. However, effluxes of up to 25 pmol cm^{-2} s^{-1} were measured in worms that had been pre-incubated in saline containing 150 mmol l^{-1} NaCl and 20 mmol l^{-1} $CaCl_2$ for 1–2 h. Effluxes were found primarily within a zone of ~75 µm anterior or posterior to the site of maximal efflux (Fig. 2D). Efflux declined to a mean value of 0.63±0.29 pmol cm^{-2} s^{-1} at distances of >100 µm posterior to the site of maximal efflux.

Our SIET measurements thus provide direct evidence for localized efflux of K^+, H^+, Na^+ and Ca^{2+} at a site on one side of the worm just anterior to the pharynx. Since the duct leading from the excretory cell opens through a pore located just anterior to the pharynx on the ventral surface of the animal (WormAtlas, www.wormatlas.org), our working hypothesis is that the effluxes described in the Results are the output of the excretory cell. These measurements thus provide the first direct evidence for ion excretion from the excretory cell of *C. elegans*. Previous studies have inferred the contributions of the cell to ionoregulation by worm swelling or mortality in response to variations in bathing medium ionic milieu when the functioning of the excretory cell was impaired by laser ablation (Nelson and Riddle, 1984).

In addition to the finding that ion effluxes are highly localized, we have also shown that efflux is enhanced when the worms are exposed to high concentrations of K^+, Na^+ or Ca^{2+} prior to measurements with SIET. Our working hypothesis is that the excretory cell contributes to homeostasis through excretion of these ions when they are present to excess. K^+ effluxes were clearly evident in worms taken directly from culture; we suggest that this reflects the high K^+ content of the bacterial food of the worms (Richey et al., 1987). Although the efflux of Na^+ efflux is localized to a site consistent with the location of the excretory pore, Na^+ influx occurs across regions of the worm posterior to the pore, suggesting that the hypodermis is a site of Na^+ uptake from the bathing medium. *C. elegans* is a soil nematode which may be exposed to Na^+-poor soil water. Under such conditions uptake of Na^+ across the hypodermis may be an important contributor to Na^+ homeostasis. The hypodermis is known to contain Na^+ transporters such as the Na^+:H^+ exchanger homolog NHX-3 (Nehrke and Melvin, 2002) and SIET measurements may be of use in future experiments to characterize the functional roles of such transporters. We detected Ca^{2+} effluxes localized to the excretory pore only after the worms had been exposed to high levels of Ca^{2+} prior to SIET measurements. Previous studies have shown that a member of the TRPM protein family, GTL-2, acts within the *C. elegans* excretory cell to mediate the excretion of excess magnesium (Teramoto et al., 2010), but much less is known of the transporters involved in excretion of excess Ca^{2+}.

In summary, we believe that SIET provides a means to measure both excretion of ions from the excretory system as well as ion transport across the hypodermis. The small size of *C. elegans* has been a barrier to physiological measurements of salt and water homeostasis. We suggest that ion-selective microelectrodes and SIET provide a means of circumventing this barrier, allowing subsequent studies to exploit the powerful molecular genetic tools available for studies of water and ion homeostasis in this model organism.

MATERIALS AND METHODS

The N2 strain of *C. elegans* was obtained from the *Caenorhabditis* Genetics Center at the University of Minnesota and was reared on *E. coli* OP50 on nematode growth medium (NGM) agar plates. Adult worms were used for all experiments. Ion fluxes out of, or into, the worms were determined using SIET. An orthogonal array of computer-controlled stepper motors fitted to a set of translator stages allow the ion-selective microelectrode to be positioned in three dimensions with submicrometer accuracy and repeatability. The microelectrode was first positioned 3–5 µm from the surface of the worm. After a wait period of 4–5 s to allow re-establishment of ion concentration gradients that had been disturbed by microelectrode movement, the microelectrode was repositioned 50 µm further out from the worm, along a line perpendicular to the long axis of the worm. The voltage at the outer site was then recorded after a further wait period, and the voltage difference between the inner and outer limits of microelectrode excursion was calculated. Hardware used for SIET measurements was obtained from Applicable Electronics (Forestdale, MA, USA) and software for control of microelectrode positioning and data acquisition was obtained from ScienceWares (Falmouth, MA, USA). The application of SIET and the construction and calibration of ion-selective microelectrodes for K^+, Na^+, H^+ and Ca^{2+} have been described previously (Donini and O'Donnell, 2005; Jayakannan et al., 2011).

Concentration gradients within the unstirred layer near the surface of the worm were determined from measurements of microelectrode voltage using the following equation:

$$\Delta C = C_B \cdot 10^{(\Delta V/S)} - C_B, \quad (1)$$

where ΔC is the concentration gradient between the two points (µmol cm^{-3}); C_B is the background ion concentration (µmol cm^{-3}), calculated as the average of the concentrations at each point; ΔV is the voltage gradient obtained from ASET (µV); and S is the slope of the electrode (µV) for a 10-fold change in ion concentration bracketing the range of interest. The concentration gradient was subsequently converted into an estimate of ion flux using Fick's first law of diffusion:

$$J_I = D_I \frac{\Delta C}{\Delta x}, \quad (2)$$

where J_I is the net flux of the ion (pmol cm^{-2} s^{-1}), D_I is the diffusion coefficient of the ion [1.55×10^{-5} cm^2 s^{-1} for Na^+, 1.92×10^{-5} cm^2 s^{-1} for K^+, 1.19×10^{-5} cm^2 s^{-1} for Ca^{2+}, 9.4×10^{-5} cm^2 s^{-1} for H^+) (Donini and O'Donnell, 2005) and Δx is the distance between the two points measured (cm).

To permit SIET measurements of ion concentration gradients in the unstirred layer near the surface of the worm, locomotion was inhibited by inclusion of the cholinergic agonist levamisole (0.5 mmol l^{-1}) in the bathing saline. Levamisole induces rigid paralysis by overstimulation of the body wall muscle. Approximately 50% of the worms had stopped moving and could be used for experiments within 30 min of levamisole treatment. In most animals, small movements of the mouth or pharyngeal pumping were observed throughout the period of exposure to levamisole.

Detection of localized changes in ion concentration by SIET was facilitated by bathing the worms in saline containing 150 mmol l^{-1} N-methyl-D-glucamine (NMDG) chloride, which does not interfere with Na^+- or K^+-selective microelectrodes. The saline also contained 1 mmol l^{-1} $MgCl_2$ and a low concentration of the ion of interest (0.15 mmol l^{-1} K^+, 0.15–3 mmol l^{-1} Na^+, 0.01 mmol l^{-1} Ca^{2+}, 10^{-7} mol l^{-1} H^+). Saline for H^+, Na^+ and K^+ measurements contained 1.8 mmol l^{-1} $CaCl_2$. Saline osmolality is close to that of M9 buffer (~300 mosmol kg^{-1}) commonly used for *C. elegans*. Worms continued to move for >12 h in saline in the absence of levamisole. Saline was buffered with 5 mmol l^{-1} Hepes for Na^+ and K^+ measurements. Buffering capacity was reduced in H^+ flux measurements by lowering the Hepes concentration to 1 mmol l^{-1}. H^+ fluxes were corrected for the effects of the buffer using equations published previously (Messerli et al., 2006). PIPES was used as a buffer for Ca^{2+} measurements because it does not form complexes with divalent metals such as Ca^{2+} (Yu et al., 1997). In all cases, worms were rinsed in 150 mmol l^{-1} NMDG and 5 mmol l^{-1} Hepes to remove bacteria and adherent fluids before transfer to the Petri dish used for SIET measurements.

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Competing interests
The authors declare no competing financial interests.

Author contributions
A.A., D.W. and M.J.O. designed the experiments. A.A. and M.J.O. collected and analyzed the data. A.A., D.W. and M.J.O. wrote the manuscript.

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