Report

Oscillatory Transepithelial H+ Flux Regulates a Rhythmic Behavior in *C. elegans*

Jason Pfeiffer,[1] David Johnson,[1] and Keith Nehrke[1,*]

[1]Department of Medicine
Nephrology Division
University of Rochester Medical Center
Rochester, New York 14642

Summary

In *C. elegans*, rhythmic defecation is timed by oscillatory Ca^{2+} signaling in the intestine [1–5]. Here, by using fluorescent biosensors in live, unrestrained worms, we show that intestinal pH also oscillates during defecation and that transepithelial proton movement is essential for defecation signaling. The intestinal cytoplasm is acidified by proton influx from the lumen during defecation. Acidification is predicted to trigger Na^+/H^+ exchange activity and subsequent proton efflux. The Na^+/H^+ exchanger NHX-7 (PBO-4) extrudes protons across the basolateral membrane and is necessary for both acute acidification of the pseudocoelom and for strong contractions of the posterior body wall muscles during defecation. This suggests that secreted protons transmit a signal between the intestine and muscle. NHX-2 is a second Na^+/H^+ exchanger whose distribution is limited to the apical membranes facing the intestinal lumen. RNA interference of *nhx-2* reduces the basal pH of the intestinal cells, reduces the rate of proton movement between the lumen and the cytoplasm during defecation, and extends the defecation period. Thus, the cell may integrate both pH and calcium signals to regulate defecation timing. Overall, these results establish the defecation cycle as a model system for studying transepithelial proton flux in tissues that maintain systemic acid-base balance.

Results and Discussion

Intracellular pH Oscillations in the Intestine Parallel Defecation

The *C. elegans* defecation cycle is an ultradian behavior that occurs rhythmically at ∼45 s intervals [6] and is timed by oscillatory Ca^{2+} signaling in the intestine [1]. The timing of defecation relies critically on the inositol-1,4,5-trisphophate receptor ($InsP_3R$), an intracellular Ca^{2+} release channel [1]. Mutations in the $InsP_3R$ itself or in upstream signaling processes that regulate $InsP_3$ production can influence the defecation period [2, 7]. The three steps of the defecation motor program (DMP) consist of a posterior body wall muscle contraction (pBoc), anterior body wall muscle contraction (aBoc), and expulsion. These steps are temporally coordinated by an intestinal Ca^{2+} wave that initiates in the posterior-most ring of the 20 epithelial cells that comprise the nematode intestine and propagates forward [3–5]. Mutations in *inx-16*, a gap junction protein [5], and *egl-8*, coding for phospholipaseC-β [4], alter the site of Ca^{2+} wave initiation. When the waves in these mutants travel through the posterior intestine in reverse (i.e., from anterior to posterior),

*Correspondence: keith_nehrke@urmc.rochester.edu

focal contractions of the posterior body wall muscles have been noted, suggesting point-to-point secretion of a signaling molecule by the intestinal epithelia [4, 5].

A reduction in the expression level of the Na^+/H^+ exchanger gene *nhx-7* by RNA interference (RNAi) resulted in a weak pBoc (data not shown). Na^+/H^+ exchangers play an important role in regulating pH and Na^+ homeostasis by mediating the electroneutral counter-transport of extracellular Na^+ and intracellular H^+. To test the hypothesis that changes in pH may be involved in defecation and particularly in the execution of pBoc, we expressed a pH-sensitive reporter, pHluorin [8], in the 20 epithelial cells of the nematode intestine. Fluorescent imaging was then used to measure intestinal intracellular pH (pH_i) in live behaving worms during defecation. The transgenic worms execute the DMP normally while being imaged and their defecation period is not significantly different than nontransgenic worms (Table S1 available online).

The pH_i of the intestinal cells begins to decrease immediately preceding contraction of the posterior body wall muscles, and the duration of the intracellular acidification persists over the entire period that the muscles are contracted (Figure 1A). The pH_i oscillations occur with the same frequency as the DMP (Figure 1B; with a period of 44.3 s versus 49.7 s; not statistically different). Imaging of pH_i oscillations in worms fed the vital pH-indicator dye BCECF confirms this observation (Figure 1C). On average, the pH_i fluctuates by ∼0.4 pH units during defecation, with pH_i recovery quickly after acidification (Figure S1A). The recovery process results in an apparent "overshoot" and a slower rate of acidification that occurs between cycles, as well. Table S1 contains average values (±standard deviations) and statistical comparisons for the period, resting pH, and amplitude of these oscillations, as well as values obtained with the other biosensors and mutants described below.

Spatially, the acidification initiates in the posterior intestinal cells and propagates through the entire intestine in a posterior-to-anterior direction in less than 1 s (Figure 1A; Figure S1B). In comparison, the Ca^{2+} wave rate is 340 μm/s and progresses through the intestine in less than a second [3–5]. The pH_i oscillation period is extended in an $InsP_3R$ mutant with an extended defecation period (Figure 1D), whereas a Ca^{2+}/calmodulin-dependent serine/threonine kinase type II (CamKII) mutant that can exhibit dual muscle contractions in a single cycle [9] also exhibits tandem pH_i oscillations (Figure 1E). These observations suggest that the decline in pH_i is controlled by a Ca^{2+}-driven pacemaker.

H+ Extrusion via the Na+/H+ Exchanger NHX-7 Signals pBoc

Na^+/H^+ exchange activity is enhanced by H^+ binding to an allosteric regulatory site on cytoplasmic C terminus of the antiporter and thus Na^+/H^+ exchangers are rapidly activated in response to cellular acidification [10]. The *nhx-7* (*pbo-4*) gene codes for a Na^+/H^+ exchanger present at the basolateral membrane specifically in the posterior intestinal cells [11]. A deletion mutation in *nhx-7* that is expected to result in a null allele reduces pBoc force (Figure S2A) without affecting rhythmic Ca^{2+} oscillations in the intestine, the defecation period (Table S1), or downstream motor components of the DMP. The
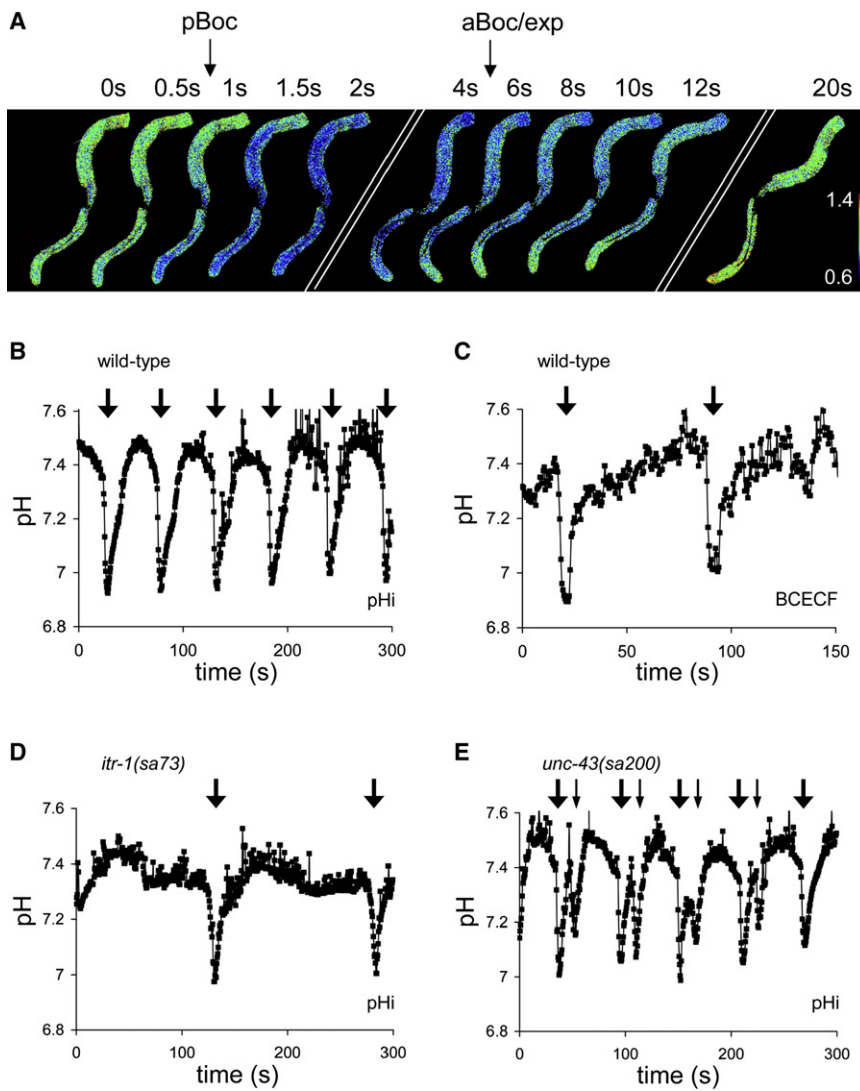
Figure 1. Intestinal pHi Dynamics in Wild-Type, *itr-1(sa73)*, and *unc-43(sa200)* Adult Animals

(A) Composite of individual frames extracted from a time-lapse acquisition of intestinal pHi in a transgenic worm expressing P*nhx-2*::pHluorin during a single round of defecation. The frames are not consecutive and the times at which they were extracted are indicated (in seconds), as are the execution of pBoc and aBoc. The posterior end of the intestine is oriented toward the bottom of each frame and the anterior end toward the top. The fluorescent ratios obtained imaging live worms (410 nm/470 nm dual excitation, 535 nm emission) have been mapped to a rainbow palette, as shown to the right. Blue corresponds to a more acidic pHi (ratio of 0.6) and red to a more alkaline pHi (ratio of 1.4).

(B–E) Representative traces of intestinal pHi oscillations derived from fluorescent imaging of live, behaving worms. The pHluorin emission ratio was converted to pH via a high K^+/nigericin calibration technique [21]. The large arrows at the top of the traces represent execution of the DMP. (B) Control worm imaged over 300 s. Table S1 contains the mean period, resting pHi, and amplitude of these oscillations from multiple worms. (C) Wild-type worm fed the pH-sensitive vital dye BCECF (490 nm/440 nm excitation, 535 nm emission), which was taken up mainly by the anterior cells of the intestine. Similar results were obtained from three separate worms. (D and E) *itr-1(sa73)* (D) and *unc-43(sa200)* (E) mutant worms, respectively. The small arrows in (E) denote where a reiteration of the principle defecation motor program occurs that result in tandem muscle contractions. All worms were imaged live and unrestrained on seeded agarose plates.

posterior body wall muscle contraction (pbo) deficit can be rescued by an extrachromosomal array expressing an NHX-7 translational GFP reporter (Figure S2). These observations led to the hypothesis that H^+ efflux across the basolateral membrane via NHX-7 is necessary for pBoc.

Intestinal pHi oscillations in the *nhx-7(ok583)* deletion mutant resemble controls (Figures 2A and 2B; mutant provided courtesy of the *C. elegans* Gene Knockout Consortium). Recovery from intracellular acidification is indistinguishable, as well (Figure S3). Thus, NHX-7 does not play an important role in intestinal pHi homeostasis. To measure pseudocoelomic pH (pHo), pHluorin was targeted to the external face of the basolateral membrane in the posterior intestinal cells via a chimeric translational fusion to the PAT-3 integrin. The distribution of the pHo reporter was confirmed by confocal micrography (Figure S4). Moreover, when the cuticle of the worm was sliced gently to allow the intestine to protrude, the fluorescence of the pHo reporter was intrinsically sensitive to buffer pH, consistent with an extracellular, membrane-anchored distribution (Supplemental Experimental Procedures).

By using the pHo biosensor, we found that the pseudocoelom surrounding the posterior intestinal cells is acutely acidified during contraction of the posterior body wall muscles

(Figure 2C). The acidification persists for approximately 3 s (Figure 2D), which is roughly the duration of pBoc (Figure S2). Acidification of the pseudocoelom is severely reduced in the *nhx-7(ok583)* mutant (Figure 2E; Table S1). These results suggest that NHX-7 causes an acute but temporally limited efflux of H^+ from the intestine.

The weak residual pHo oscillations and contractions that remain in the *nhx-7(ok583)* mutant might reflect a response to cellular acidification by other nhx proteins residing in the intestinal basolateral membrane [11]. Rescue of these weak contractions in the *nhx-7(ok583)* background via transgene rescue strongly suggest that acute H^+ extrusion across the basolateral membrane occurs predominantly via NHX-7 (Figure S2). However, it is possible that slower transport mechanisms at the basolateral membrane might contribute to pHi recovery in the cells, but that the H^+ diffuse quickly through the pseudocoelom relative to their extrusion rate without inducing an acute or detectable pHo transient. These data demonstrate that H^+ are a critical component of the signal secreted by the intestine that triggers contraction of the body wall muscles. It remains unclear whether Ca^{2+} signaling is required for NHX-7 activity during defecation or whether acidification of the intestinal cells is sufficient to cause H^+ efflux via an allosteric effect of H^+ binding to NHX-7.
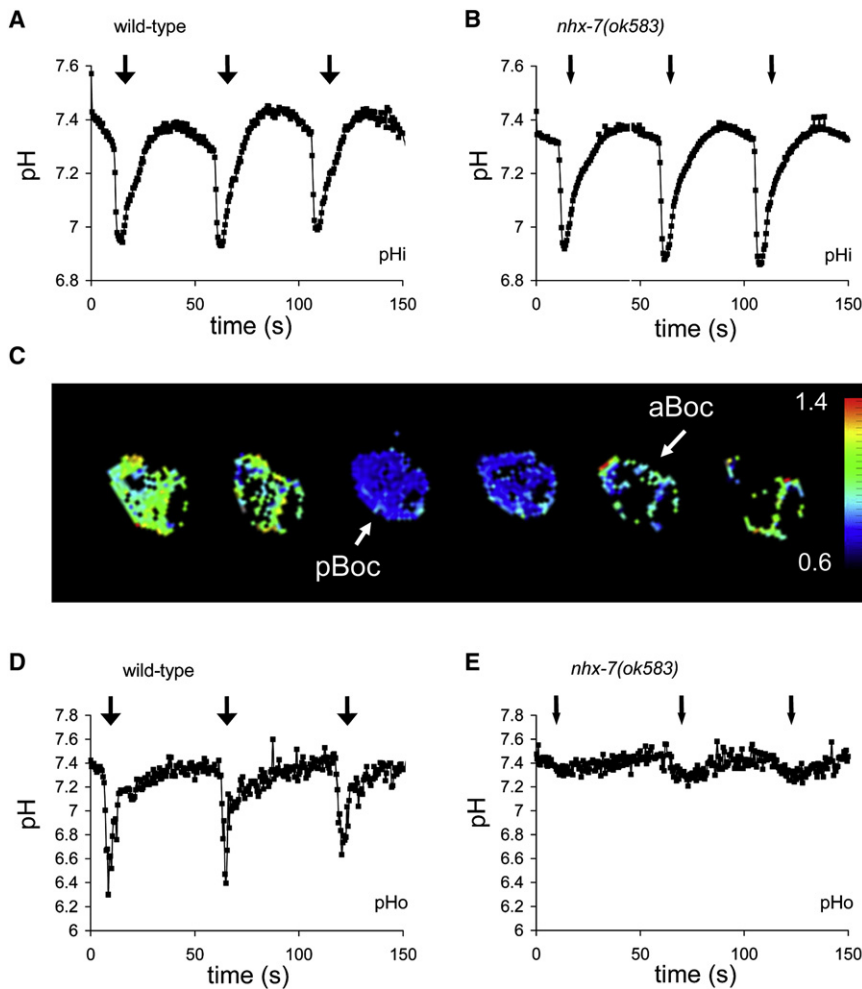
Figure 2. NHX-7 Regulates Extracellular, but Not Intracellular, pH Dynamics

Wild-type (A, C, D) and *nhx-7(ok585)* mutants (B, E).

(A and B) Representative traces of intestinal pHi oscillations during defecation obtained with the P*nhx-2*::pHluorin biosensor in live, behaving worms.

(C) Composite of individual frames extracted from an acquisition series of ratiometric images (nonconfocal) obtained in live worms expressing an extracellular pHo P*nhx-7*::PAT-3::pHluorin biosensor (410 nm/470 nm dual excitation, 535 emission, every 1.5 s) in the posterior-most intestinal cells. The ratiometric images were pseudo-colored with a rainbow palette, as shown, with blue representing low pH (ratio of 0.6) and red representing high pH (ratio of 1.4). The changes in color reflect pHo oscillations in the pseudocoelom surrounding the posterior-most intestinal ring. The arrow depicting pBoc is pointed from the posterior toward the anterior of the intestine. The arrow depicting aBoc is pointed from the anterior toward the posterior. These arrows reflect the movement of the contents of the intestinal lumen that occur during the respective muscle contractions.

(D and E) Representative traces of pHo oscillations during defecation in control (D) and *nhx-7(ok583)* mutants (E). Weak pBoc are denoted by small arrows, and normal pBoc are indicated by larger arrows above the traces. Please see Table S1 for average values and statistical significance.

H⁺ Enter the Cell from the Intestinal Lumen

Because both the cytoplasm and pseudocoelom are acidified during defecation, we reasoned that the lumen may be the source of H^+ driving this process. To test this hypothesis, we measured the pH of the lumen (pHl) by feeding worms dextran conjugated to the pH-sensitive vital dye Oregon Green 488 in S-basal media for 1 hr followed by live imaging on plates (Figure 3A). The intestinal lumen rests at a pH value of ~4.1 in wild-type worms and, consistent with our hypothesis, rises quickly to ~6.0 prior to returning to an acidic resting pH in a rhythmic series of oscillations that coincide with defecation (Figure 3B; Table S1). So why does the luminal H^+ concentration change by nearly 2000 times that of the cytoplasm during defecation? One possibility is that the luminal volume may be much less than the volume of cytoplasm, reflecting the fact that pH is a function of H^+ concentration. A second is that the cytoplasm may contain more buffering capacity than the lumen. A third possibility is that a unidirectional transepithelial flux of acid equivalents occurs; however, our data argue against this being strictly the case (Figure 2B), and luminal pH oscillations resemble controls in the *nhx-7(ok583)* mutant (Table S1).

In fact, these results suggest that the bulk of the intracellular H^+ load both enters and exits the cell through the apical membrane, with only a limited efflux of H^+ through NHX-7 being necessary to trigger pBoc (Figure 2B). Perhaps the direction of H^+ transport through the apical membrane is determined by Ca^{2+} signaling? Regardless, we predict that H^+ transport

into the intestinal cells during defecation prevents their loss to the environment as the luminal contents are expelled, and their return to the lumen preserves a gradient across the apical membrane that is used to drive H^+-coupled nutrient uptake.

The Na⁺/H⁺ Exchanger NHX-2 Modulates Defecation Timing and H⁺ Flux at the Apical Membrane

The Na⁺/H⁺ exchanger NHX-2 is targeted to the apical membrane facing the intestinal lumen [11]. Reducing the expression of *nhx-2* by ~85%–90% by RNAi (as determined by Q-PCR) has been shown to reduce the resting pHi of the intestinal cytoplasm [12] and doubles the average defecation period from ~44 s to 86 s (Table S1). Defecation timing is thought to be mainly a function of Ca^{2+} signaling, and regulation of defecation timing by an acid-base transporter may highlight a unique connection between pH and Ca^{2+} signaling processes.

The pH oscillation periods obtained from live, unrestrained worms after *nhx-2* RNAi were calculated from fluorescent imaging experiments. These periods are ~81 s in the transgenic pHi strain, ~95 s in the transgenic pHo strain, and ~71 s in worms fed dextran-conjugated Oregon Green 488 (Table S1), which are comparable to the extension caused by *nhx-2* RNAi in control nematodes. The fact that the defecation behavior occurs coincident with each pH oscillation in individual worms strongly suggests that the slight variations noted in oscillation periods are a function of either the biosensors or the heterogeneity inherent in RNAi.
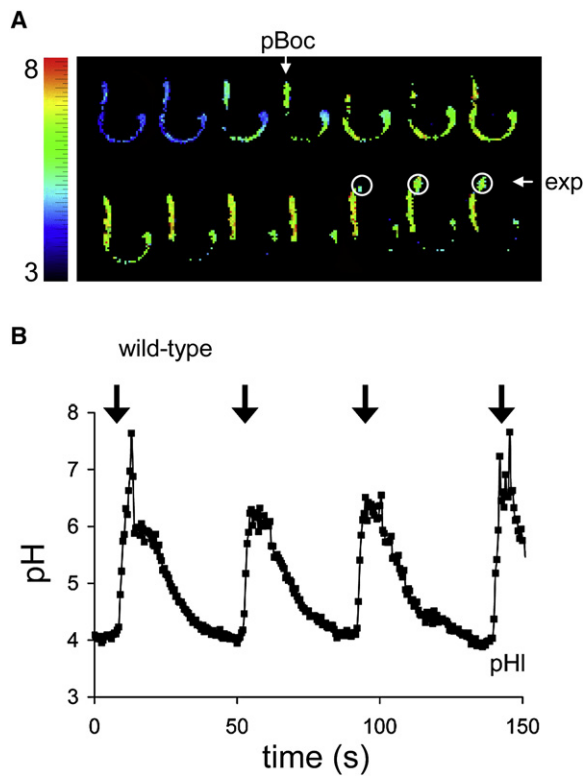
Figure 3. Luminal Alkalinization during Defecation

(A) Representative frames extracted from a time-lapse acquisition of luminal pH (pHl) oscillations during defecation. Consecutive frames obtained at 2 Hz are shown. Wild-type worms were fed dextran coupled to the pH-sensitive vital dye Oregon Green 488. The fluorescent ratios (490 nm/440 nm dual excitation, 535 nm emission) obtained by imaging live worms during defecation were mapped to a rainbow palette, as indicated. The execution of pBoc and explusion are denoted. For orientation, the posterior end of the intestine is near the expelled luminal contents, encompassed by white circles. The plate is ~pH 6. In general, because of loading efficiency and retention of the dye, the worms used to obtain pHl measurements were larval rather than adult animals.

(B) Representative trace of pHl oscillations during defecation in wild-type worms. The arrows denote pBoc. The ratios obtained from Oregon Green 488 imaging were converted to pH. Transient spikes in pHl above 6 are likely artifacts; as the ratio increases beyond the linear dynamic range of the calibration curve, small changes in ratio lead to progressively larger apparent changes in pH. A full description of this data can be found in Table S1.

The resting intestinal pHi is ~7.2 in the *nhx-2(RNAi)* worms (Figure 4A; Table S1), as noted previously [12], but the intestinal pHo lies ~4.1 (Figure 4B; Table S1), which is not significantly different from controls. However, the magnitude of both the pHi and pHl oscillations is reduced by loss of NHX-2 (Figures 4A and 4B; Figure S5 and Table S1). These data suggest that NHX-2 contributes to H$^+$ movement through the apical membrane, either directly or indirectly.

The apparently decreased rates of H$^+$ flux across the apical membrane might be expected to translate into a decreased rate of H$^+$ extrusion across the basolateral membrane, resulting in a pbo phenotype. But surprisingly we found that oscillations in pHo were of normal amplitude and were actually increased in duration (Figure 4C). These data were confirmed by visually observing luminal compression as a result of posterior body wall muscle contractions; *nhx-2(RNAi)* worms had significantly longer contractions than did wild-type worms (Figure S2B). A reduced influx of H$^+$ may be offset in part by
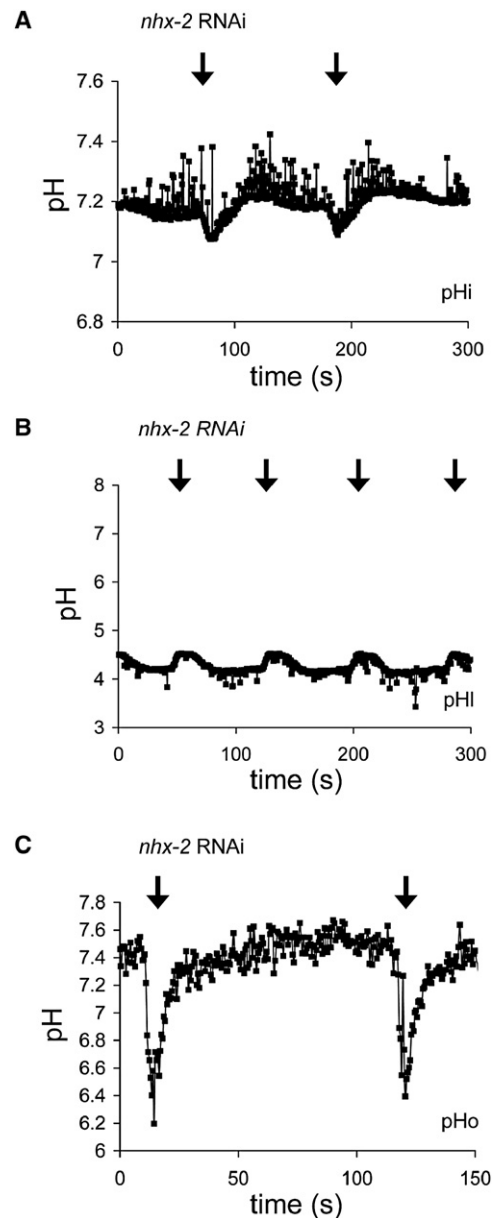

Figure 4. pH Dynamics in Worms Treated with *nhx-2* RNAi

Representative traces after the loss of NHX-2 show (A) intestinal pHi oscillations in a live worm expressing a P*nhx-2*::pHluorin biosensor, (B) pHl oscillations in a live worm fed Oregon Green 488, and (C) intestinal pHo oscillations in a live worm expressing a P*nhx-7*::PAT-3::pHluorin biosensor. Table S1 contains average values and a statistical analysis of significance.

the acidic resting pHi; because the pHi is more acidic to begin with, the nadir during pHi oscillations reaches 7.06, which is not significantly different from control worms (Table S1). This may be sufficient to elicit H$^+$ efflux via NHX-7, resulting in strong body wall muscle contractions. However, because NHX-7 activity has little effect on pHi, it is unlikely that even the increased efflux observed here will result in significant cellular alkalinization.

Finally, we examined Ca^{2+} and pHi wave dynamics after loss of NHX-2 (Figure S6). Apart from the extended cycle period, the only significant difference appeared to be a slower rate of Ca^{2+} clearance in the anterior-most intestinal cells. Although the pHi

oscillations are reduced in amplitude, the anterior cells of the intestine begin to acidify within 1 s of the posterior cells, reflecting similar wave dynamics as in the controls (Figure S6D and S6F).

Several conclusions arise from these data. First, the H^+ gradient between the cytoplasm and the lumen is not established solely as a function of NHX-2, which is consistent with theory (i.e., antiport activity is gradient driven). Given the magnitude of the pH change that occurs in the lumen and the observation that H^+ extrusion from the cell into the lumen must eventually take place against a large pH gradient, energy-dependent transporters such as V-ATPases are likely to be involved. Second, the normal flow of H^+ between the lumen and the cytoplasm requires NHX-2. This could reflect direct transport by NHX-2 acting in reverse mode to allow H^+ into the cell or alternatively through an indirect mechanism where the actual H^+ conduit is inhibited by the reduced pHi that accompanies loss of NHX-2. Finally, both pBoc and basolateral H^+ extrusion are enhanced after the loss of NHX-2, which, while counterintuitive, may reflect the reduced resting pHi in the cells. This observation may also highlight a role for Ca^{2+} oscillations in activating NHX-7, and our unpublished observations suggest that the carboxyl terminus of NHX-7 contains a high-affinity calmodulin binding site.

Our work establishes the *C. elegans* defecation cycle as a model for cyclic transepithelial H^+ secretion and systemic acid-base transport in secretory epithelia. We have identified H^+ as a component of the signal secreted by the intestinal epithelia that is necessary for contraction of the posterior body wall muscles. Na^+/H^+ exchangers are ubiquitously expressed in both worms and mammals, and proton secretion via this class of protein may represent a novel mode of cell-cell communication. Recent work by Erik Jorgensen's group (published while this manuscript was under review) used a genetic approach to identify *nhx-7* and showed that pHo oscillations were both necessary and sufficient to induce pBoc [13]. Furthermore, their work identified a cys-loop-type nicotinic acetylcholine receptor expressed in posterior body wall muscles that is activated by low pH and is necessary for pBoc, as well. The identification of this receptor, combined with a vertebrate family of H^+-sensing G protein coupled receptors (PSR) with widespread tissue distribution [14, 15], suggests that H^+ signaling may be more commonplace than previously thought. Our data confirm that H^+ can act as a transmitter for muscular contractions and provide a mechanism for the secretion of H^+ through cyclic intracellular acidification.

Our results also suggest a functional interplay between Ca^{2+} signaling and acid-base transport. There are many well-established mechanisms through which pH can alter Ca^{2+} signaling. The binding of InsP$_3$ to its receptor is pH sensitive [16], as is the gating of TRPM-type cation channels [17]; interestingly, the TRPM family members *gtl-1* and *gon-2* regulate electrolyte homeostasis in the intestine of worms [18]. pHi may also govern physiologic processes that occur downstream of oscillatory Ca^{2+} signaling such as Ca^{2+} waves. In fact, both pHi and Na^+/H^+ exchange activity in mammalian acinar cells modifies Ca^{2+} wave propagation [19]. Moreover, both Ca^{2+} and pH play a role in chemical gating of gap junction channels that allow the passage of small molecules between cells [20]. In the defecation model, H^+ gating of gap junctions might prevent cellular metabolites from moving "backwards" through the intestine and help to shape the Ca^{2+} wave. In conclusion, our results suggest that interplay between two signaling molecules implement and condition a rhythmic motor program in an intact, behaving animal.

References

1. Dal Santo, P., Logan, M., Chisholm, A., and Jorgensen, E. (1999). The inositol triphosphate receptor regulates a 50-second behavioral rhythm in *C. elegans*. Cell *98*, 757–767.
2. Espelt, M., Estevez, A., Yin, X., and Strange, K. (2005). Oscillatory Ca^{2+} signaling in the isolated *Caenorhabditis elegans* intestine: role of the inositol-1,4,5-trisphosphate receptor and phospholipases C {beta} and {gamma}. J. Gen. Physiol. *126*, 379–392.
3. Teramoto, T., and Iwasaki, K. (2006). Intestinal calcium waves coordinate a behavioral motor program in *C. elegans*. Cell Calcium *40*, 319–327.
4. Nehrke, K., Denton, J., and Mowrey, W. (2007). Intestinal Ca^{2+} wave dynamics in freely moving *C. elegans* coordinate execution of a rhythmic motor program. Am. J. Physiol. Cell Physiol. *294*, 333–344.
5. Peters, M.A., Teramoto, T., White, J.Q., Iwasaki, K., and Jorgensen, E.M. (2007). A calcium wave mediated by gap junctions coordinates a rhythmic behavior in *C. elegans*. Curr. Biol. *17*, 1601–1608.
6. Thomas, J. (1990). Genetic analysis of defecation in *Caenorhabditis elegans*. Genetics *124*, 855–872.
7. Norman, K., Fazzio, R., Mellem, J., Espelt, M., Strange, K., Beckerle, M., and Maricq, A. (2005). The Rho\/Rac-family guanine nucleotide exchange factor VAV-1 regulates rhythmic behaviors in *C. elegans*. Cell *123*, 119–132.
8. Miesenbock, G., De Angelis, D.A., and Rothman, J.E. (1998). Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature *394*, 192–195.
9. Liu, D., and Thomas, J. (1994). Regulation of a periodic motor program in *C. elegans*. J. Neurosci. *14*, 1953–1962.
10. Aronson, P.S., Nee, J., and Suhm, M.A. (1982). Modifier role of internal H^+ in activating the Na^+/H^+ exchanger in renal microvillus membrane vesicles. Nature *299*, 161–163.
11. Nehrke, K., and Melvin, J.E. (2002). The NHX family of Na^+/H^+ exchangers in *Caenorhabditis elegans*. J. Biol. Chem. *277*, 29036–29044.
12. Nehrke, K. (2003). A reduction in intestinal cell pHi due to loss of the *Caenorhabditis elegans* Na^+/H^+ exchanger NHX-2 increases life span. J. Biol. Chem. *278*, 44657–44666.
13. Beg, A.A., Ernstrom, G.G., Nix, P., Davis, M.W., and Jorgensen, E.M. (2008). Protons act as a transmitter for muscle contraction in *C. elegans*. Cell *132*, 149–160.
14. Ludwig, M.G., Vanek, M., Guerini, D., Gasser, J.A., Jones, C.E., Junker, U., Hofstetter, H., Wolf, R.M., and Seuwen, K. (2003). Proton-sensing G-protein-coupled receptors. Nature *425*, 93–98.
15. Ishii, S., Kihara, Y., and Shimizu, T. (2005). Identification of T cell death-associated gene 8 (TDAG8) as a novel acid sensing G-protein-coupled receptor. J. Biol. Chem. *280*, 9083–9087.
16. White, A.M., Varney, M.A., Watson, S.P., Rigby, S., Liu, C.S., Ward, J.G., Reese, C.B., Graham, H.C., and Williams, R.J. (1991). Influence of Mg^{2+} and pH on n.m.r. spectra and radioligand binding of inositol 1,4,5-trisphosphate. Biochem. J. *278*, 759–764.
17. Andersson, D.A., Chase, H.W.N., and Bevan, S. (2004). TRPM8 activation by menthol, icilin, and cold is differentially modulated by intracellular pH. J. Neurosci. *24*, 5364–5369.

18. Teramoto, T., Lambie, E.J., and Iwasaki, K. (2005). Differential regulation of TRPM channels governs electrolyte homeostasis in the *C. elegans* intestine. Cell Metab. *1*, 343–354.

19. Gonzalez, A., Pfeiffer, F., Schmid, A., and Schulz, I. (1998). Effect of intracellular pH on acetylcholine-induced Ca^{2+} waves in mouse pancreatic acinar cells. Am. J. Physiol. Cell Physiol. *275*, C810–C817.

20. Peracchia, C. (2004). Chemical gating of gap junction channels: roles of calcium, pH and calmodulin. Biochim. Biophys. Acta *1662*, 61–80.

21. Thomas, J.A., Buchsbaum, R.N., Zimniak, A., and Racker, E. (1979). Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry *18*, 2210–2218.