

## Cationic pathway of pH regulation in larvae of *Anopheles gambiae*

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### SUMMARY

*Anopheles gambiae* larvae (*Diptera: Culicidae*) live in freshwater with low Na<sup>+</sup> concentrations yet they use Na<sup>+</sup> for alkalinization of the alimentary canal, for electrophoretic amino acid uptake and for nerve function. The metabolic pathway by which larvae accomplish these functions has anionic and cationic components that interact and allow the larva to conserve Na<sup>+</sup> while excreting H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup>. The anionic pathway consists of a metabolic CO<sub>2</sub> diffusion process, carbonic anhydrase and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers; it provides weak HCO<sub>3</sub><sup>-</sup> and weaker CO<sub>3</sub><sup>2-</sup> anions to the lumen. The cationic pathway consists of H<sup>+</sup> V-ATPases and Na<sup>+</sup>/H<sup>+</sup> antiporters (NHAs), Na<sup>+</sup>/K<sup>+</sup> P-ATPases and Na<sup>+</sup>/H<sup>+</sup> exchangers (NHEs) along with several (Na<sup>+</sup> or K<sup>+</sup>):amino acid<sup>+/-</sup> symporters, a.k.a. nutrient amino acid transporters (NATs). This paper considers the cationic pathway, which provides the strong Na<sup>+</sup> or K<sup>+</sup> cations that alkalinize the lumen in anterior midgut then removes them and restores a lower pH in posterior midgut. A key member of the cationic pathway is a Na<sup>+</sup>/H<sup>+</sup> antiporter, which was cloned recently from *Anopheles gambiae* larvae, localized strategically in plasma membranes of the alimentary canal and named AgNHA1 based upon its phylogeny. A phylogenetic comparison of all cloned NHAs and NHEs revealed that AgNHA1 is the first metazoan NHA to be cloned and localized and that it is in the same clade as electrophoretic prokaryotic NHAs that are driven by the electrogenic H<sup>+</sup> F-ATPase. Like prokaryotic NHAs, AgNHA1 is thought to be electrophoretic and to be driven by the electrogenic H<sup>+</sup> V-ATPase. Both AgNHA1 and alkalophilic bacterial NHAs face highly alkaline environments; to alkalinize the larva mosquito midgut lumen, AgNHA1, like the bacterial NHAs, would have to move nH<sup>+</sup> inwardly and Na<sup>+</sup> outwardly. Perhaps the alkaline environment that led to the evolution of electrophoretic prokaryotic NHAs also led to the evolution of an electrophoretic AgNHA1 in mosquito larvae. In support of this hypothesis, antibodies to both AgNHA1 and H<sup>+</sup> V-ATPase label the same membranes in *An. gambiae* larvae. The localization of H<sup>+</sup> V-ATPase together with (Na<sup>+</sup> or K<sup>+</sup>):amino acid<sup>+/-</sup> symporter, AgNAT8, on the same apical membrane in posterior midgut cells constitutes the functional equivalent of an NHE that lowers the pH in the posterior midgut lumen. All NATs characterized to date are Na<sup>+</sup> or K<sup>+</sup> symporters so the deduction is likely to have wide application. The deduced colocalization of H<sup>+</sup> V-ATPase, AgNHA1 and AgNAT8, on this membrane forms a pathway for local cycling of H<sup>+</sup> and Na<sup>+</sup> in posterior midgut. The local H<sup>+</sup> cycle would prevent unchecked acidification of the lumen while the local Na<sup>+</sup> cycle would regulate pH and support Na<sup>+</sup>:amino acid<sup>+/-</sup> symport. Meanwhile, a long-range Na<sup>+</sup> cycle first transfers Na<sup>+</sup> from the blood to gastric caeca and anterior midgut lumen where it initiates alkalinization and then returns Na<sup>+</sup> from the rectal lumen to the blood, where it prevents loss of Na<sup>+</sup> during H<sup>+</sup> and HCO<sub>3</sub><sup>-</sup> excretion. The localization of H<sup>+</sup> V-ATPase and Na<sup>+</sup>/K<sup>+</sup>-ATPase in *An. gambiae* larvae parallels that reported for *Aedes aegypti* larvae. The deduced colocalization of the two ATPases along with NHA and NAT in the alimentary canal constitutes a cationic pathway for Na<sup>+</sup>-conserving midgut alkalinization and de-alkalinization which has never been reported before.

Key words: proton pump, H<sup>+</sup> V-ATPase, sodium pump, Na<sup>+</sup>/K<sup>+</sup> P-ATPase, cation exchanger, NHE, NHA, AgNHA1, (Na<sup>+</sup> or K<sup>+</sup>):amino acid<sup>+/-</sup> symporter, nutrient amino acid transporter, NAT, AgNAT8, African malaria mosquito.

### INTRODUCTION

The pH of the luminal contents in mosquito larvae increases from near neutrality in the foregut to a value that exceeds 10 in anterior midgut then drops to 7.5 in the posterior midgut (Dadd, 1975; Ramsay, 1950). The alkaline environment is optimal for mosquito digestive enzymes, sterilizes food and dissociates the tannin-protein complexes that are ingested in the plant detritus diet of the larvae. A similar alkalinization pattern in the anterior midgut of the caterpillar, *Manduca sexta*, is partially understood and serves as a model for the alkalinization process. In caterpillars, the apical membrane of the midgut cells is hyperpolarized by an H<sup>+</sup> V-ATPase and the positive voltage drives a putative K<sup>+</sup>/2H<sup>+</sup> antiporter (Wieczorek et al., 1991). The antiporter provides strong K<sup>+</sup> cations as counter-ions for the weak carbonate anions (>50 mmol l<sup>-1</sup>) in the

lumen (Turbeck et al., 1968) (for reviews, see Harvey and Wieczorek, 1997; Wieczorek et al., 1999) which together generate the high pH.

Any hypothesis for midgut alkalinization in fresh water mosquito larvae must take into account the observations (1) that the larvae live in a Na<sup>+</sup>-poor environment, and (2) that they excrete (rather than exhale) metabolic CO<sub>2</sub> (del Pilar Corena et al., 2004; Stobbart, 1971). In mosquito larval midgut as in caterpillars the principal anions controlling pH in the alimentary canal are HCO<sub>3</sub><sup>-</sup> and CO<sub>3</sub><sup>2-</sup> (Boudko et al., 2001a); but unlike caterpillars in which the Na<sup>+</sup> concentration is very low, the principal cations in mosquito larval alimentary canal are Na<sup>+</sup> and K<sup>+</sup> (Clements, 1992). The key question is: how are Na<sup>+</sup> and K<sup>+</sup> accumulated in the anterior midgut lumen then removed in the posterior midgut and hindgut with the

result that  $\text{Na}^+$  is retained while  $\text{H}^+$  and  $\text{HCO}_3^-$  are excreted (Strange et al., 1982; Strange and Phillips, 1984; Strange et al., 1984).

It is thought that the high alkalinity in larval mosquito anterior midgut is achieved *via* two interacting pathways. In the anionic pathway metabolic  $\text{CO}_2$  diffuses from cells into the ectoperitrophic space all along the alimentary canal where it is hydrated to  $\text{HCO}_3^-$  by an extracellular carbonic anhydrase (Smith et al., 2007). An anion exchanger in the gastric caeca is postulated to remove  $\text{Cl}^-$  from the lumen in exchange for  $\text{HCO}_3^-$  (Boudko et al., 2001a). This present paper considers the cationic pathway, which consists of  $\text{H}^+$  V-ATPases,  $\text{Na}^+/\text{K}^+$  P-ATPases, ( $\text{Na}^+$  or  $\text{K}^+$ ) antiporters and exchangers (NHAs and NHEs, respectively) and ( $\text{Na}^+$  or  $\text{K}^+$ ) nutrient amino acid symporters (a.k.a. NATs). Our working hypothesis is that alkalization in anterior midgut is achieved through the action of electrophoretic NHAs that use voltage gradients ( $\Delta\Psi$ ) generated by  $\text{H}^+$  V-ATPases to replace lumen  $\text{H}^+$  with  $\text{Na}^+$ . The return to nearly neutral pH in posterior midgut is achieved by novel, functional NHEs comprising  $\text{H}^+$  secreting  $\text{H}^+$  V-ATPases linked to  $\text{Na}^+$ -absorbing NATs (Boudko et al., 2005b), which we call  $\text{NHE}_{\text{V-NAT}}$ s. These *de facto* exchangers have the same orientation as eukaryotic NHEs. Perhaps  $\text{NHE}_{\text{V-NAT}}$ s are part of the reason why there are only three NHEs in insect genomes compared to nine NHEs in mammalian genomes (Orlowski and Grinstein, 2004).

In this work we localized  $\text{H}^+$  V-ATPase and  $\text{Na}^+/\text{K}^+$  P-ATPase with respect to a newly cloned  $\text{Na}^+$ -coupled amino acid transporter, AgNAT8 (Meleshkevitch et al., 2006) as a representative NAT. We also localized a newly cloned NHA from the malaria mosquito *Anopheles gambiae* and deduced that it is electrophoretic on the grounds that it usually is located in the same membrane sector as the electrogenic  $\text{H}^+$  V-ATPase (Table 1). Although the relative locations of  $\text{H}^+$  V-ATPase and  $\text{Na}^+/\text{K}^+$ -ATPase have been described in *Aedes aegypti* larva (Patrick et al., 2006), their locations relative to NHAs and NATs to form a cationic pathway have never been reported before.

## MATERIALS AND METHODS

### Procurement and rearing of mosquitoes

*Anopheles gambiae* Giles *sensu stricto* (Diptera, Culicidae, G3 strain) eggs were obtained from the Malaria Research and Reference Reagents Resource Center (MR4) of the Center for Disease Control and Prevention (CDC) in Atlanta, Georgia, USA. The eggs were hatched in distilled water at  $28\pm 2^\circ\text{C}$  and kept in a securely locked incubator. After hatching, the larvae were subdivided into small mosquito breeder containers (Bioquip® Products, Rancho Dominguez, CA, USA) each holding approximately 200 larvae in 400 ml of distilled water. The larvae were maintained according to standard rearing procedures developed at the Whitney Laboratory under the guidance of the CDC, and have been approved by the Bureau of Entomology and Pest Control of the Florida Department of Agriculture and Consumer Services. The larvae were fed freshly ground TetraMin™ tropical flakes mixed with baker's yeast every 2 days. Development was carefully monitored until the early fourth instar stage when larvae were used in experiments.

### Labeling midgut *in vivo* with pH sensitive dye

The pH gradient along the midgut was visualized in an immobilized, intact *An. gambiae* larva that had ingested a 0.1% solution of m-Cresol Purple dye (Fig. 1). The image was captured with a CCD camera and processed with CorelDRAW Graphics Suite X3®.

### Procurement of antibodies

Four antibodies were used to localize  $\text{H}^+$  V-ATPase,  $\text{Na}^+/\text{K}^+$ -ATPase, AgNHA1 and AgNAT8 in mosquito larval alimentary canal. The  $\text{H}^+$  V-ATPase antibody was raised in a rabbit against the B subunit of the  $\text{H}^+$  V-ATPase enzyme of *Culex quinquefasciatus* (Filippova et al., 1998), the amino acid sequence of which is 98% identical to the *An. gambiae* protein. This antibody has been used successfully to localize the  $\text{H}^+$  V-ATPase in *Ae. aegypti* (Patrick et al., 2006). The  $\text{Na}^+/\text{K}^+$ -ATPase monoclonal antibody was obtained from The Developmental Studies Hybridoma Bank, University of Iowa, IA, USA and is reactive to a variety of insect species including mosquitoes (Patrick et al., 2006). It was raised against a chicken kidney antigen and recognizes the  $\alpha$ -subunit of the  $\text{Na}^+/\text{K}^+$ -ATPase. The AgNHA1 antibody was produced by immunizing rabbits with two synthetic peptides, one from the N-terminal region (FSEALEKIERDYDNSRL) and the other from the extracellular loop between transmembrane domains 11 and 12 (LKTVMSENRTTEEVHY) (Rheault et al., 2007). The antibody labels a single band on a western blot (Fig. 2). The AgNAT8 antibody was also raised in a rabbit to recognize a unique 19 amino acid sequence (CGPIDPATHYEEKKFIDED) located on the carboxylic acid terminus of the AgNAT8 protein; this sequence is specific to AgNAT8 although the western blot shows more than one band, perhaps because of multimerization.

### Extraction of *Anopheles gambiae* membranes

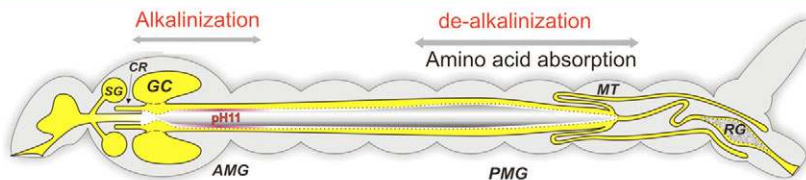
Larval mosquito tissues were isolated and membrane fractions were collected for use in western blot analysis. Briefly, early fourth instar larvae were placed in ice-cold  $50\text{ mmol l}^{-1}$  Tris-HCl with a cocktail of protease inhibitors. The larvae were homogenized with 30 strokes of a Dounce homogenizer. The resulting crude homogenate was centrifuged at  $3000\text{ g}$  at  $4^\circ\text{C}$  for 10 min to remove debris and then the supernatant was centrifuged at  $10\,000\text{ g}$  at  $4^\circ\text{C}$  for 10 min to remove mitochondria. The resulting supernatant, containing the membranes, was centrifuged at  $100\,000\text{ g}$  at  $4^\circ\text{C}$  for 1 h using a Beckman SW 41 Ti rotor. The pellet was resuspended in the homogenization buffer and its protein content estimated using Bio-Rad reagents (Bradford, 1976). Aliquots of the membrane suspension were stored in a  $-20^\circ\text{C}$  freezer until used for western analysis.

### Western blots

The frozen mosquito membrane aliquots were thawed and treated with NuPAGE® LDS sample loading buffer (Invitrogen Life Technologies, CA, USA). Mosquito membrane protein ( $25\text{ }\mu\text{g}$ ) was loaded on a 4–12% Bis-Tris polyacrylamide gel and electrophoresed under reducing conditions for 30 min at 150 V and 120 mA. Then they were electro-transferred onto a  $0.45\text{ }\mu\text{m}$  pore size nitrocellulose membrane (Millipore, Billerica, MA, USA) at 30 V for 1 h at  $4^\circ\text{C}$  using the X-Cell tank transfer system (Invitrogen Life Technologies, CA, USA). The transferred proteins were visualized by staining with 0.1% Fast Green for 1 min. For western blot analysis, the nitrocellulose membrane was blocked with buffer containing 2.5% non-fat dry milk powder (Carnation®) in Tris-buffered saline (TBS) containing 0.2% Tween 20 (TBST) for 1 h at room temperature. The nitrocellulose membranes were then incubated overnight at  $4^\circ\text{C}$  with the antibodies to  $\text{H}^+$  V-ATPase (1:2000),  $\text{Na}^+/\text{K}^+$ -ATPase (1:50), AgNHA1 (1:1000) and AgNAT8 (1:1000) diluted in blocking buffer. As a control, lanes with identical membrane concentrations of AgNHA1 and AgNAT8 were incubated with pre-immunization sera. After washing in TBST, the nitrocellulose membranes were



Fig. 1. A living *An. gambiae* larva that was fed m-Cresol Purple dye illustrates the well known anterior to posterior pH gradient along the larval mosquito alimentary canal. The pH is mildly alkaline in gastric caeca (GC), increases to high values in the anterior midgut (AMG), starts to drop in central midgut, returns to mildly alkaline in posterior midgut (PMG) and becomes neutral at the posteriormost region of the hindgut. SG, salivary gland; CR, cardia; MT, Malpighian tubule; RG, rectum.



incubated with alkaline phosphatase-coupled goat anti-rabbit and/or anti-mouse antibody (Jackson ImmunoResearch Lab, PA, USA) at a dilution of 1:2000 in 1% blocking buffer for 2 h at room temperature. After four rinses in TBS for 15 min each, antibody binding to the proteins was visualized by the alkaline phosphate color precipitation procedure.

#### Immunolabeling of whole mounts

To prepare whole mounts, fourth instar larvae were immobilized in ice-cold  $0.1 \text{ mol l}^{-1}$  phosphate-buffered saline (PBS) solution and then pinned at the head and tail to a Petri dish lined with Silastic E RTV (Dow Corning, USA). The cuticle was opened using microscissors and pinned back at the corners to expose the whole alimentary canal, which was fixed in 4% paraformaldehyde in  $0.1 \text{ mol l}^{-1}$  PBS (PFA) for 2 h, rinsed three times for 30 min each in  $0.1 \text{ mol l}^{-1}$  PBS and incubated in 0.3% Triton X-100 in PBS (PBT) for 6–12 h. Next, the larvae were incubated in a blocking solution of 1% bovine serum albumin (BSA) in PBT for 12–24 h at  $4^\circ\text{C}$ . Then the antibodies were added at a dilution of 1:1000 for  $\text{H}^+$  V-ATPase, 1:10 for  $\text{Na}^+/\text{K}^+$ -ATPase and 1:250 for AgNHA1 and AgNAT8 and the larvae were incubated at  $4^\circ\text{C}$  on a shaker for a further 12 h. After washing in PBT they were incubated overnight at  $4^\circ\text{C}$  in fluorescent-labeled secondary antibody at a dilution of 1:800 in  $0.1 \text{ mol l}^{-1}$  PBS. After rinsing, the alimentary canal was dissected away from the carcass, mounted in 60% glycerol in  $0.1 \text{ mol l}^{-1}$  PBS and examined with a Leica laser scanning confocal microscope (LSCM).

#### Immunolabeling of sections

To prepare thin paraffin sections, procedures were the same as those used for whole mounts up to the isolation and fixation steps. The fixed and isolated alimentary canals were rinsed in PBS followed by an ethanol dehydration series of 10, 30, 50, 70, 90 and 100%. They were then incubated in a 70:30 mixture of ethanol:xylene followed by 30:70 ethanol:xylene for 1 h each before a final incubation in 100% xylene overnight at room temperature. The following day, finely chopped paraffin chips (Paraplast Plus™) were added to the now dehydrated mosquito guts to make a 50:50 v/v mixture of paraffin to xylene. After 3–4 h, the xylene was refreshed and the samples kept overnight at room temperature. They were transferred to an oven at  $56^\circ\text{C}$  until the paraffin melted, when

half the volume in the vials was replaced with fresh melted paraffin and the incubation continued for 2–3 h with several replacements of the paraffin. The larval tissues in melted paraffin were transferred to an embedding mold and allowed to solidify within fresh paraffin. Sections ( $6 \mu\text{m}$  thick) of the larval alimentary canal were cut on an American Optical Rotatory microtome – Model # 820 (Buffalo, NY, USA) and mounted onto glass slides coated with a solution of 1% gelatin in 0.1% chromium potassium sulfate. Prior to staining with antibody the sections were cleared of wax in 100% xylene, rehydrated through an ethanol series (100, 90, 70, 50, 30 and distilled water) and finally washed in PBT. The slides were then blocked in a solution of 1% BSA in PBT for 1 h at room temperature and incubated at  $4^\circ\text{C}$  overnight with the antibodies at dilutions of 1:1000 for  $\text{H}^+$  V-ATPase, 1:10 for  $\text{Na}^+/\text{K}^+$ -ATPase, 1:250 for AgNHA1. Frozen AgNAT8 sections were used for immunolabeling using standard methods. Briefly, the fixed gut was incubated in 30% sucrose in  $0.1 \text{ mol l}^{-1}$  PBS for 12 h. After embedding in TissueTek® (Sakura FineTek USA Inc., Torrance, CA, USA), the gut was frozen at  $-30^\circ\text{C}$  and  $15 \mu\text{m}$  sections were cut on a Leica cryostat and mounted on glass slides. The sections were rehydrated in PBS for 20 min and then permeabilized in PBT for 20 min, before the immunolabeling step. Non-specific binding sites were blocked using a solution composed of 2% normal goat serum (NGS), 1% BSA in 0.3% Triton X-100 in  $0.1 \text{ mol l}^{-1}$  PBS for 2 h. After rinsing in PBT for 15 min four times, the AgNAT8 antibodies were applied at a dilution of 1:50. The sections (both paraffin and frozen) were rinsed with blocking solution, and incubated with secondary antibody conjugated to fluorophores for 3 h at room temperature. Finally, the sections were rinsed in blocking solution and mounted in a solution of glycerol–gelatin–Tris at a pH of 7.4 and enclosed with coverslips. To visualize nuclei, a DNA dye DRAQ 5 (Biostatus Limited, Shephed, UK) was applied at a dilution of 1:1000. The sections were examined on a Leica LSCM and the captured images processed using CorelDRAW Graphics Suite X3.

## RESULTS

### Midgut pH gradient

The pH is alkaline throughout the alimentary canal of mosquito larvae. In *Ae. aegypti* the gastric caecal pH is 7.6–8.5, in anterior midgut it is 9.7–10, and in central midgut (a.k.a. transitional zone)



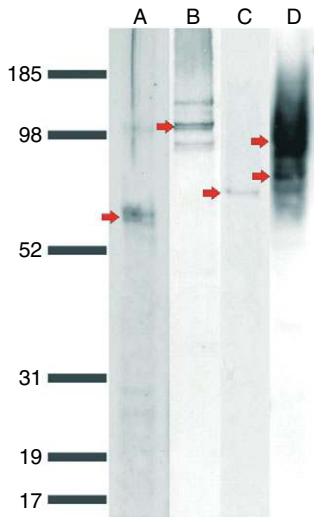


Fig. 2. Western blots of mosquito larval membrane proteins separated on SDS-PAGE and probed with a polyclonal antibody to subunit B of the  $H^+$  V-ATPase (A), a monoclonal antibody to subunit  $\alpha$  of the  $Na^+/K^+$  P-ATPase (B), and polyclonal epitope-specific antibodies to AgNHA1 (C) and AgNAT8 (D). The arrows indicate the band of protein that is recognized by each antibody. The nitrocellulose membrane was cut into strips and probed with the different antibodies leading to differences in the backgrounds of the blots.

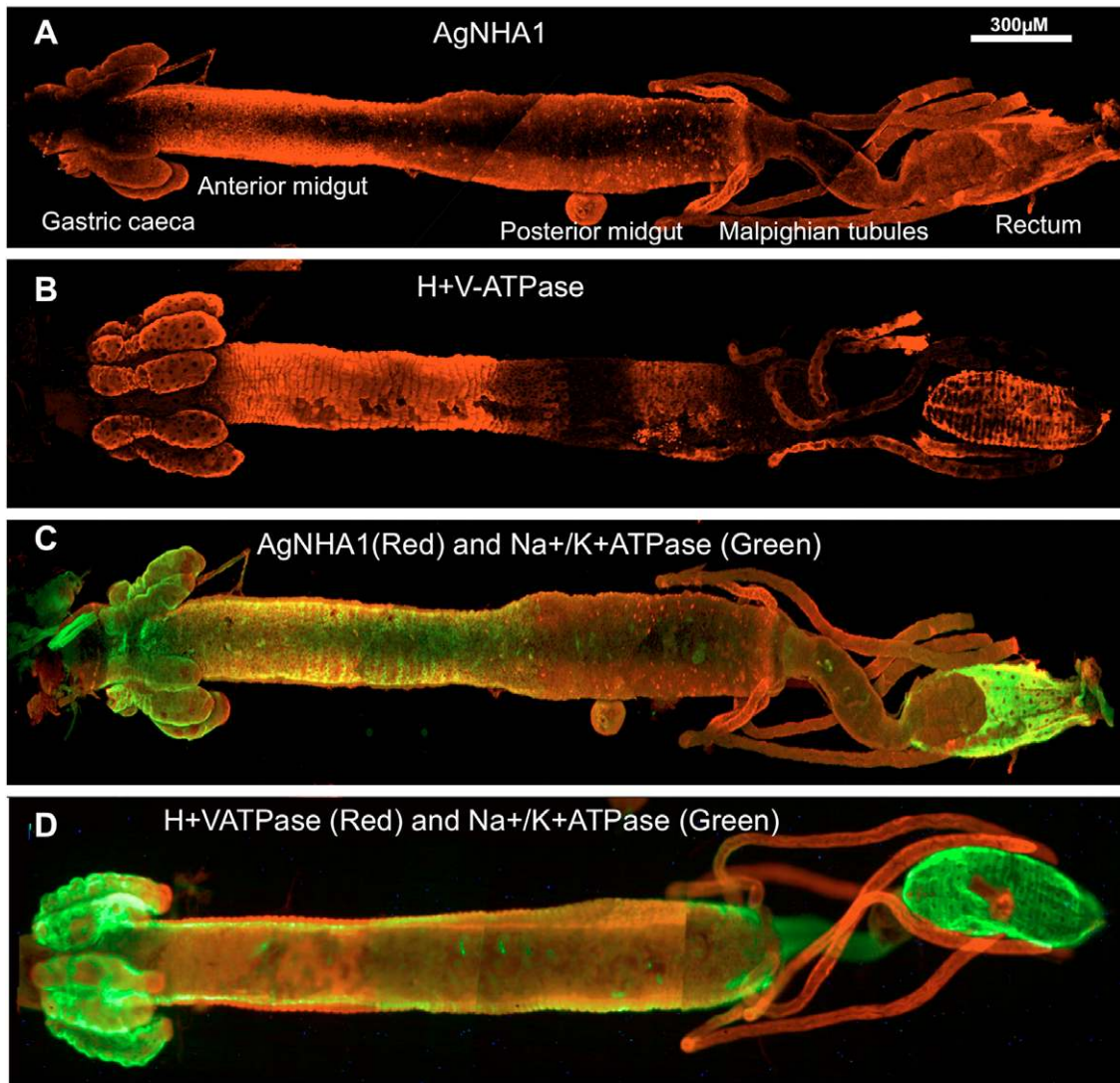


Fig. 3. Max projection images of immunolocalized transport proteins in the alimentary canal of larval *An. gambiae*. (A,B) The localization of AgNHA1 (red) and  $H^+$  V-ATPase (red), respectively. (C) The colocalization of AgNHA1 (red) with  $Na^+/K^+$ -ATPase (green). (D) The colocalization of  $H^+$  V-ATPase (red) with  $Na^+/K^+$  P-ATPase (green). Ventral view of the alimentary canal so DAR cells are not visible. The yellow color in C,D results from the colocalization of AgNHA1 and  $H^+$  V-ATPase (both red) with  $Na^+/K^+$  P-ATPase (green).

it drops from 10.1 to 9.3 and continues to drop from 9.0 to 7.0 in posterior midgut. Finally in the hindgut the pH stabilizes at values between 6.5 and 7.5 (Clements, 1992; Ramsay, 1950). More recent measurements (Boudko et al., 2001b) fall within the range of these earlier values. The anterior–posterior pH profile in *An. gambiae* larval midgut as revealed by a pH sensitive dye is similar (Fig. 1).

#### Specificity of antibodies

The reactivity and specificity of the four antibodies that had been demonstrated previously in several mosquitoes was confirmed in western blots of larval *An. gambiae* membranes (Fig. 2). Three of the antibodies recognized a single band within the expected range of molecular mass: H<sup>+</sup> V-ATPase subunit B ~60 kDa (lane A), Na<sup>+</sup>/K<sup>+</sup> P-ATPase subunit  $\alpha$  ~110 kDa (lane B) and AgNHA1 ~72 kDa (lane C). The western blot for the antibody to AgNAT8 labeled a band at ~70 kDa (lane D). The images have been converted to 8 bit gray-scale using ImageJ software (Abramoff et al., 2004)

to correct for the background differences due to the different antibodies used (Fig. 2).

#### Immunolocalization of AgNHA1, H<sup>+</sup> V-ATPase, Na<sup>+</sup>/K<sup>+</sup>-ATPase

In whole mounts of larval alimentary canal, antibodies to AgNHA1 intensely labeled the anterior midgut region, proximal portions of the Malpighian tubules and rectum (Fig. 3A). The antibodies to H<sup>+</sup> V-ATPase intensely labeled the posterior cells of the gastric caeca, the anterior midgut, the Malpighian tubules and the rectum (Fig. 3B). In colocalization images of whole alimentary canal, the labeling pattern for AgNHA1 (red) contrasted sharply with that for Na<sup>+</sup>/K<sup>+</sup>-ATPase (green, Fig. 3C). This sharp contrast was also seen in the colocalization images of H<sup>+</sup> V-ATPase (red) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (green; Fig. 3D). In the gastric caeca, the AgNHA1 (red; Fig. 3D) and H<sup>+</sup> V-ATPase (red; Fig. 3D) antibody labeled the posterior cells. In the anterior midgut, AgNHA1 (red) and H<sup>+</sup> V-ATPase (red) appeared to be on the blood-side whereas Na<sup>+</sup>/K<sup>+</sup>-ATPase (green) was toward the lumen-side (Fig. 3C and 3D, respectively). The small

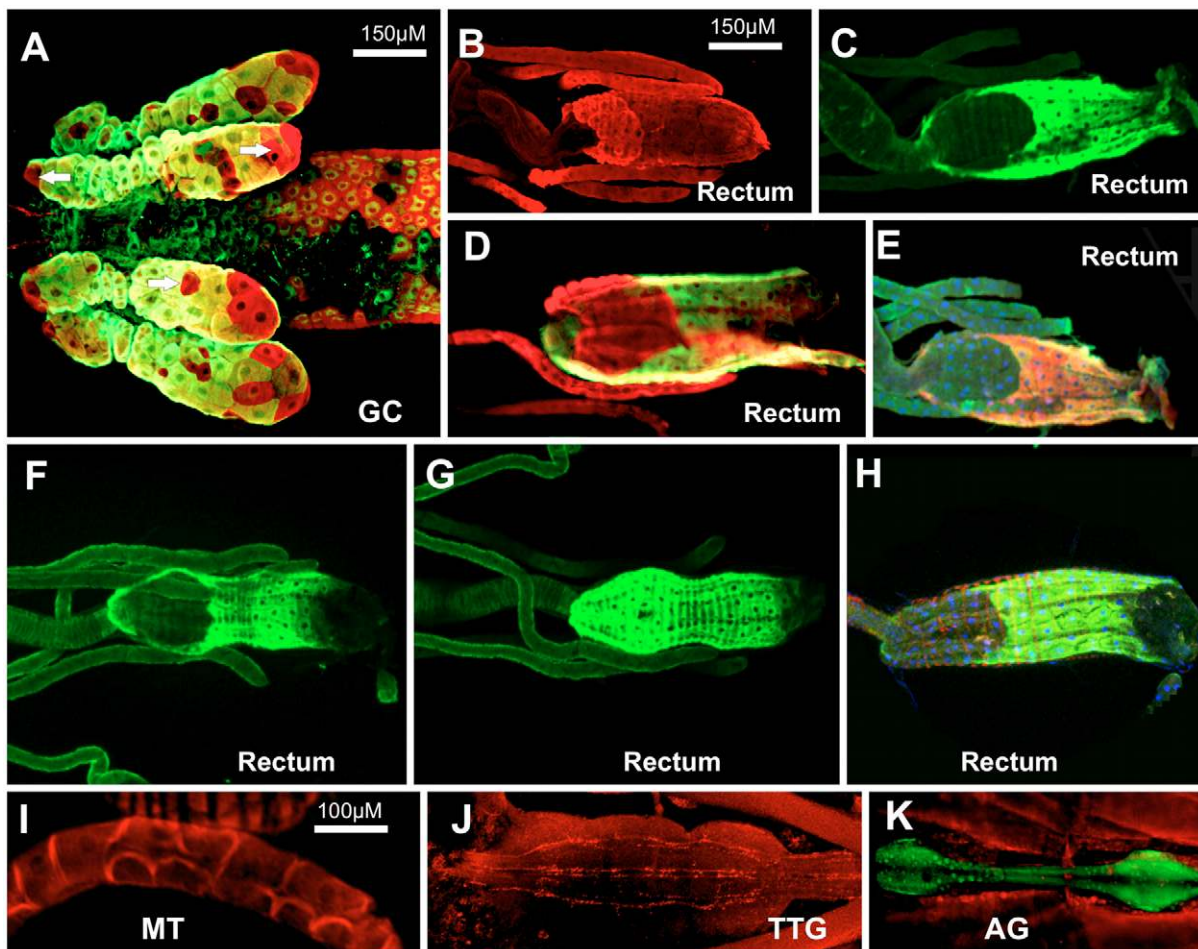


Fig. 4. Max projection images at higher magnification than in Fig. 3 of regions of the alimentary canal showing the location of transport proteins. (A) Posterior cap cells and other scattered cells (white arrows) in the gastric caeca are labeled intensely with the H<sup>+</sup> V-ATPase antibody (red) whereas the rest of the caecal cells are stained with Na<sup>+</sup>/K<sup>+</sup> P-ATPase antibody (green). (B) The DAR cells are labeled with H<sup>+</sup> V-ATPase antibody (red). (C) The rest of the rectum is labeled with Na<sup>+</sup>/K<sup>+</sup> P-ATPase antibody (green). (D) H<sup>+</sup> V-ATPase (red) and Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green) are colocalized in the rectum. (E) AgNHA1 (red) and Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green) are also colocalized in the rectum; the DAR cells have a greenish background. (F, H) Na<sup>+</sup>/K<sup>+</sup> P-ATPase is present on the dorsal side of the rectum with the conspicuous absence of staining in the DAR cells. (G) In a ventral view of the rectum, Na<sup>+</sup>/K<sup>+</sup> P-ATPase labeling (green) is widespread. (A, D, E) The yellow color results from the colocalization of AgNHA1 or H<sup>+</sup> V-ATPase (both red) with Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green). (I) H<sup>+</sup> V-ATPase is present on the apical membranes of the principal cells in the Malpighian tubules (MT). (J, K) AgNHA1 and the Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green) are present in the entire nervous system. Shown here are the trilobed thoracic ganglion (TTG; J) and abdominal ganglion (AG; K). Scale bars in A and B (for B–H), 150  $\mu$ m; in I (for I–K), 100  $\mu$ m.



Table 1. Location of components of cationic pathway

Region	H <sup>+</sup> V-ATPase	Na <sup>+</sup> /K <sup>+</sup> P-ATPase	AgNHA1	AgNAT8	pH
Foregut	Unknown	Unknown	Unknown	Not detected	7.5
Cardia	Unknown	Unknown	Apical vesicles	Basal	7.5
Distal gastric caecum	Apical	Basal	Possibly basal	Basal	8
Proximal gastric caecum	Apical	Basal	Possibly basal	Basal	8
Anterior midgut	Basal	Apical	Apical vesicles	Apical	10.5
Central midgut	Basal→apical	Apical→basal	Vesicles→apical	Apical	9.7
Posterior midgut	Apical	Basal	Apical	Apical	7.5
Ileum	Not detected	Not detected	Not detected	Not detected	7
Dorsal anterior rectum	Apical/cytoplasmic	Not detected	Not detected	Possibly apical	7.5
Rest of rectum	Apical	Basal	Possibly apical	Possibly apical	6.0

central midgut region between the anterior midgut and posterior midgut was labeled intensively by the Na<sup>+</sup>/K<sup>+</sup> P-ATPase antibody (Fig. 3C,D). In the posterior midgut there was both Na<sup>+</sup>/K<sup>+</sup> P-ATPase and H<sup>+</sup> V-ATPase whereas in Malpighian tubules H<sup>+</sup> V-ATPase but not Na<sup>+</sup>/K<sup>+</sup> P-ATPase was expressed (Fig. 3D).

#### Deduced colocalization of H<sup>+</sup> V-ATPase and AgNHA1

Antibodies to H<sup>+</sup> V-ATPase and AgNHA1 invariably were localized to the same regions in the alimentary canal. Since these results were not obtained from the overlay of the two antibodies, they will be

called 'deduced colocalizations'. Where the AgNHA1 (red) labeling was observed in Fig. 3C, there was strong labeling with the H<sup>+</sup> V-ATPase antibody (red) in Fig. 3D in the gastric caeca, anterior midgut, posterior midgut, the Malpighian tubules and parts of the rectum. The AgNHA1 antibody labeled cells in the tips of the gastric caecal lobes (Fig. 3C) at the same location as the H<sup>+</sup> V-ATPase labeling (Fig. 3D). These cells have been called cap cells (Corena et al., 2002); they are structurally distinct from the 'ion transporting cells' of Volkmann and Peters (Volkmann and Peters, 1989) (P.J.L., unpublished observations).

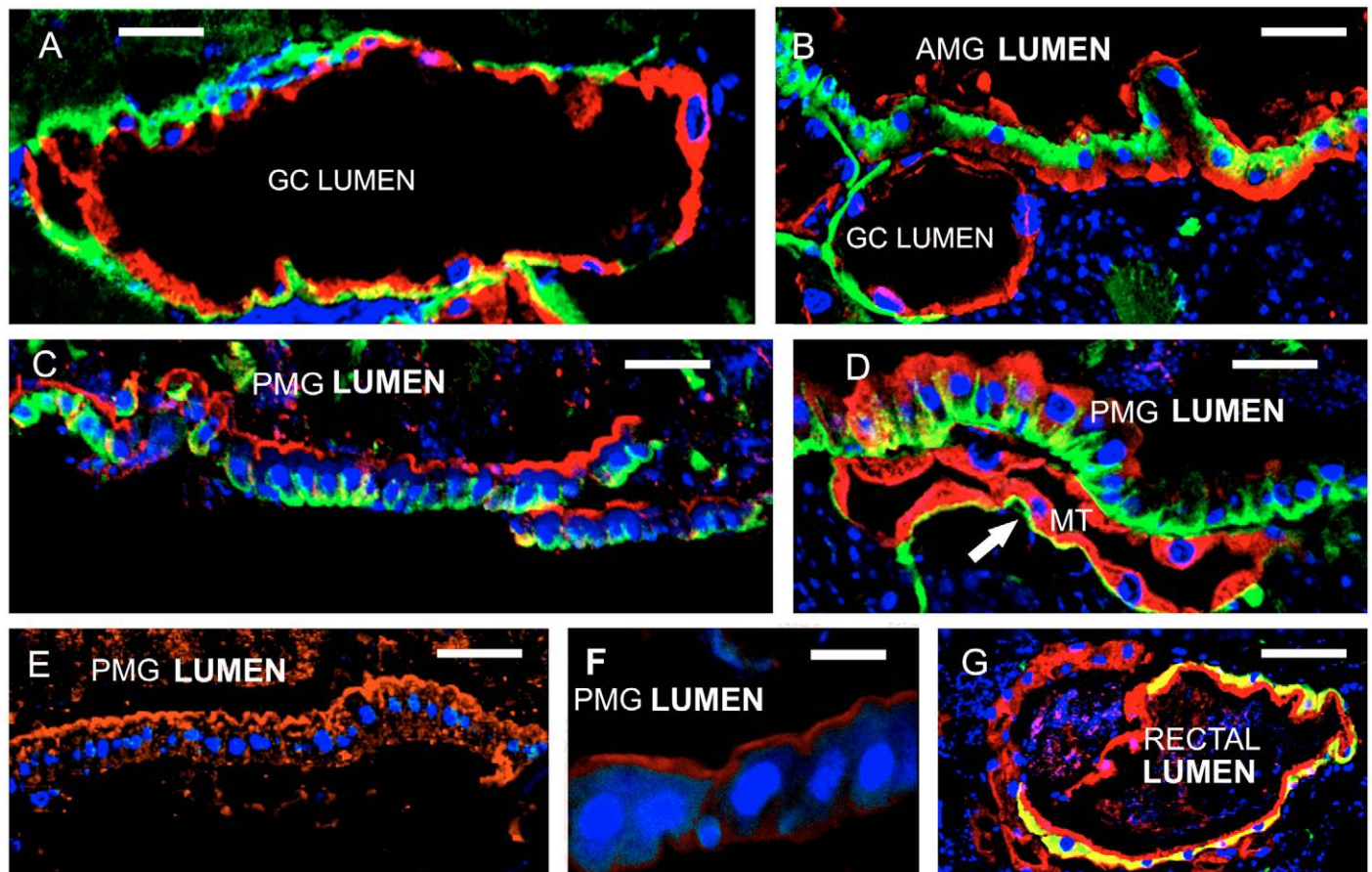


Fig. 5. Immunolocalization of transport proteins in longitudinal sections of mosquito alimentary canal from various regions. H<sup>+</sup> V-ATPase (red) and Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green) antibodies labeled sections of the gastric caeca (GC; A), anterior midgut (AMG; B), posterior midgut (PMG; C,D), a Malpighian tubule (MT; white arrow in D) and rectum (G). The apical membrane of posterior midgut region is labeled with AgNHA1 antibody (red; E) and a nutrient amino acid transporter, AgNAT8 antibody (red; F). The yellow color in A,D,G results from the colocalization of H<sup>+</sup> V-ATPase (red) with Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green). The nuclei are labeled blue with DRAQ. Scale bar, 100 μm.

Table 2. The membrane location of portosomes and V-ATPase immunostaining

Species	Region	Portosome location	Reference	V-ATPase location	Reference
<i>Culex pipiens</i>	GC	Apical in ion-transporting cell	(Clements, 1992)	Posterior caeca	(Filippova et al., 1998)
<i>Ae. aegypti</i> , <i>An. gambiae</i> , <i>C. pipiens</i> , <i>An. stephensi</i>	GC	Apical in ion-transporting cell	(Volkman and Peters, 1989)	Posterior caeca in <i>Ae. aegypti</i>	(Patrick et al., 2006)
<i>An. gambiae</i>	GC	–	–	Apical in cap cells	This work
<i>Ae. aegypti</i>	AMG	Basal membranes	(Cioffi, 1984; Zhuang et al., 1999)	Basal	(Zhuang et al., 1999; Patrick et al., 2006)
<i>An. gambiae</i>	AMG	–	–	Basal	This work
<i>Ae. aegypti</i>	PMG	Apical	(Zhuang et al., 1999)	Apical Apical	(Zhuang et al., 1999) (Patrick et al., 2006)
<i>An. gambiae</i>	–	–	–	Apical	This work
<i>Ae. taeniorhynchus</i> , <i>Culexeta inornata</i> , <i>Ae. aegypti</i>	MT	Apical in principal cells	(Clements, 1992)	Apical, <i>Ae. aegypti</i> principal cells	(Patrick et al., 2006)
<i>An. gambiae</i>	MT	–	–	Apical in principal cells	This work

GC, gastric caeca; AMG, anterior midgut; PMG, posterior midgut; MT, malpighian tubules.

At higher magnification of the whole mounts, the heterogeneous nature of the protein localizations was revealed at the cellular level (Fig. 4A–H). There was a differential expression of Na<sup>+</sup>/K<sup>+</sup> P-ATPase and H<sup>+</sup> V-ATPase in the gastric caeca (Fig. 4A) and rectum (Fig. 4D). In the gastric caeca, the H<sup>+</sup> V-ATPase was highly expressed in the cap cells and also at the proximal ends (Fig. 4A). In addition, specific cells in the medial part of the gastric caeca also strongly expressed H<sup>+</sup> V-ATPase (red) whereas the surrounding cells strongly expressed Na<sup>+</sup>/K<sup>+</sup> P-ATPase (green). This H<sup>+</sup> V-ATPase antibody labeling pattern in the cap cells of the gastric caeca was mirrored exactly by the AgNHA1 antibody labeling pattern (compare Fig. 3C with D). The Na<sup>+</sup>/K<sup>+</sup>-ATPase labeled much of the gastric caeca except for the cap cells (Fig. 4A).

#### Labeling of rectum reveals two separate regions

In the rectum of the mosquito there was intense labeling by the H<sup>+</sup> V-ATPase (red) in a subset of cells in the dorsal, anterior sector (Fig. 4B); this subset of cells is in a similar location to the rectal pads of terrestrial insects that were first described by Berlese in 1909 (see Wigglesworth, 1972) and which divide the rectum into two functional parts (Grueber and Bradley, 1994). The H<sup>+</sup> V-ATPase antibody labeled the other rectal cells with lower intensity. These cells did not label with the Na<sup>+</sup>/K<sup>+</sup>-ATPase antibody (green; Fig. 4C) or AgNHA1 antibody (red; Fig. 3A), although the rest of the rectum labeled intensely with both of them (Fig. 4E). The DAR cells in Fig. 3 have a background that is red. These cells appeared only on the dorsal side (Fig. 4F) and not the ventral side (Fig. 4G,H) of the rectum and for this reason they are called dorsal anterior rectal (DAR) cells and are also distinct in specific expression of carbonic anhydrase 9 (Smith et al., 2007).

The Malpighian tubules labeled with the H<sup>+</sup> V-ATPase antibody primarily in the apical membranes of the principal cells (Fig. 4I).

#### Labeling of nerves

Some neural tracts, neuronal cell bodies and nerve fascicles throughout the nervous system of the larval mosquito labeled intensely with AgNHA1 (Fig. 4J) and Na<sup>+</sup>/K<sup>+</sup>-ATPase (Fig. 4K) antibodies. The classical K<sup>+</sup>/Na<sup>+</sup> diffusion potentials that are secondary to the ion gradients generated by Na<sup>+</sup>/K<sup>+</sup> P-ATPases

could provide energy to drive an electrophoretic NHA. Alternatively, since 3Na<sup>+</sup> are ejected for every 2K<sup>+</sup> driven in, Na<sup>+</sup>/K<sup>+</sup> P-ATPases, like H<sup>+</sup> V-ATPases, are membrane hyperpolarizing enzymes that could energize the NHA. The precise location and role of AgNHA1 in the larval nervous system clearly merits further study.

#### H<sup>+</sup> V-ATPase labeling of portosome-containing membranes

In longitudinal paraffin sections the H<sup>+</sup> V-ATPase antibody labeling paralleled published reports of portosomes (Harvey et al., 1981), which are V<sub>1</sub>-ATPase particles (Grüber et al., 2000). Portosomes have been reported in certain microvilli of other mosquito species (Clements, 1992; Volkman and Peters, 1989; Zhuang et al., 1999). In *An. gambiae* the H<sup>+</sup> V-ATPase antibody labeled the apical membrane of the cells in the gastric caeca, posterior midgut, Malpighian tubules (Fig. 5A–D) and rectum (Fig. 5G); it also labeled the basal membrane of the anterior midgut cells (Fig. 5B). Portosomes have been observed at these sites in gastric caeca, anterior midgut, and Malpighian tubules of *Ae. aegypti* and *Culex quinquefasciatus* mosquitoes (Table 2).

#### Cell polarity of Na<sup>+</sup>/K<sup>+</sup>-ATPase and H<sup>+</sup> V-ATPase

There was intense labeling of Na<sup>+</sup>/K<sup>+</sup>-ATPase on the basal membranes of the cells of the gastric caeca except those of the cap cells (Fig. 5A). In the distal and proximal tips of the gastric caeca, labeling of H<sup>+</sup> V-ATPase was prominent but its polarization was not clear (Fig. 5A). In the anterior midgut there was intense labeling by Na<sup>+</sup>/K<sup>+</sup>-ATPase on the apical membranes whereas the H<sup>+</sup> V-ATPase antibody intensely labeled the basal membranes (Fig. 5B). Basal H<sup>+</sup> V-ATPase and apical Na<sup>+</sup>/K<sup>+</sup>-ATPase are the reverse of their classical positions in which Na<sup>+</sup>/K<sup>+</sup>-ATPase is basal and H<sup>+</sup> V-ATPase is apical. In the posterior midgut, Na<sup>+</sup>/K<sup>+</sup>-ATPase was localized on the basal membranes whereas antibodies to H<sup>+</sup> V-ATPase (Fig. 5C), AgNHA1 (Fig. 5E) and AgNAT8 (Fig. 5F) all labeled the apical membranes. In Malpighian tubules the Na<sup>+</sup>/K<sup>+</sup>-ATPase was localized on basal membranes whereas the H<sup>+</sup> V-ATPase was localized on apical membranes with some expression in the cytosol (Fig. 5D). In the rectum the Na<sup>+</sup>/K<sup>+</sup>-ATPase was present on the basal membranes and H<sup>+</sup> V-ATPase was on the apical membranes.

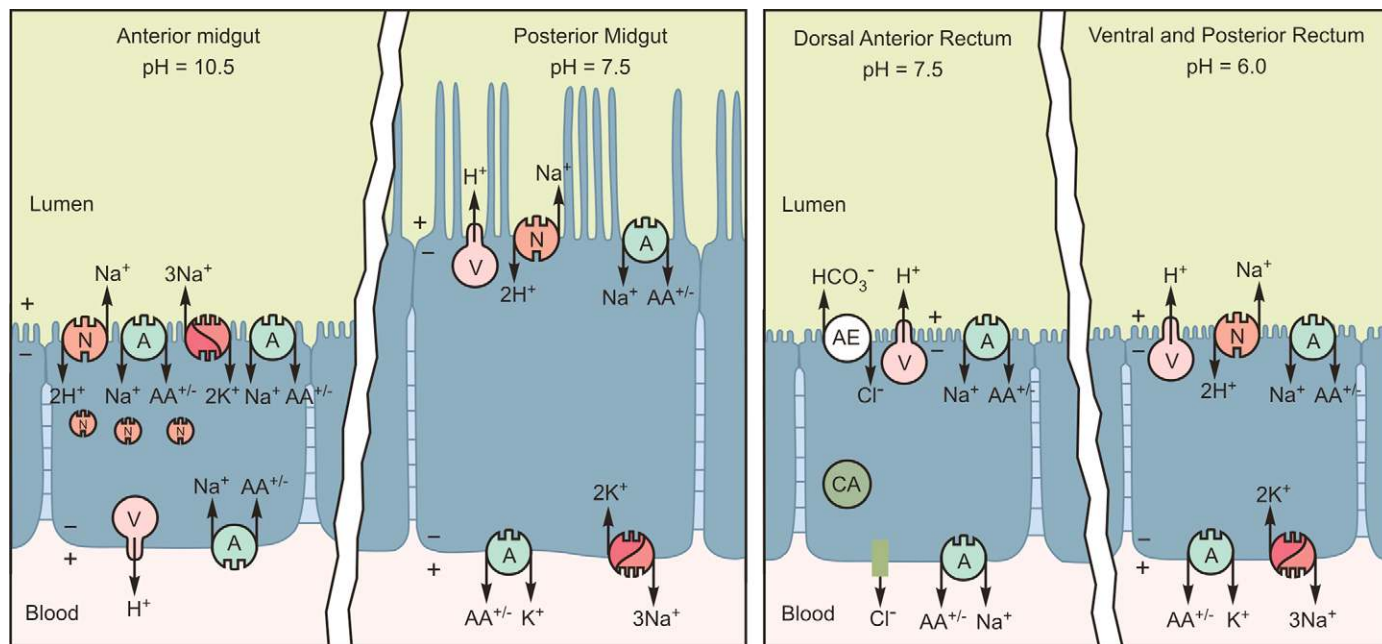


Fig. 6. Model illustrating the role of membrane transport proteins in pH regulation as well as in  $H^+$ ,  $Na^+$  and  $K^+$  recycling and amino acid absorption. The localization and colocalizations of  $H^+$  V-ATPase, AgNAT8, AgNHA1 and  $Na^+/K^+$  P-ATPase provide insight into these processes in anterior and posterior midgut cells. The rectum is divided into two functional parts based on the immunolocalization of the transport proteins; postulated interactions between these and yet to be identified proteins are discussed in the text. The shades of pink or orange indicate transport proteins localized in this study; shades of green indicate proteins localized in other studies and white indicates postulated but unidentified proteins. The key points are (1) that  $H^+$  V-ATPase is basal and  $Na^+/K^+$  P-ATPase is apical in anterior midgut, (2) that  $H^+$  V-ATPase and AgNAT8 constitute a functional NHE (NHE<sub>V-NAT</sub>) in the apical membrane of posterior midgut cells, and (3) that AgNHA1 recycles  $H^+$  in conjunction with  $H^+$  V-ATPase and recycles  $Na^+$  in conjunction with AgNAT8 in the apical membrane of posterior midgut cells.

Although  $H^+$  V-ATPase was prominent on plasma membranes of DAR cells of the rectum and also within the cytoplasm, no labeling of  $Na^+/K^+$ -ATPase was observed there (Fig. 5G).

## DISCUSSION

### NHEs and NHAs

Two types of transporters that exchange alkali metal ions for hydrogen ions across biological membranes are well known. Electroneutral  $Na^+/H^+$  exchangers (NHEs) are said to be expressed in cells of all eukaryotic organisms; they use the inwardly directed  $Na^+$  gradients that are generated by  $Na^+/K^+$  P-ATPases to drive  $Na^+$  into cells and metabolic  $H^+$  out of cells (Orlowski and Grinstein, 1997; Orlowski and Grinstein, 2004). Even better understood are the electrophoretic ( $Na^+$  or  $K^+$ )/ $H^+$  antiporters (NHAs) of alkalophilic bacteria; they use the voltage gradient generated by  $H^+$  F-ATPases to drive  $H^+$  into cells and  $Na^+$  out of cells. Several eukaryotic NHEs and bacterial NHAs have been cloned, localized and characterized (Orlowski and Grinstein, 2004; Padan et al., 2005). Indeed, a bacterial NHA has been crystallized, its ion binding sites and reaction mechanism are known (Arkin et al., 2007) and its role in pH regulation has been elucidated (Hunte et al., 2005). An enigmatic, electrophoretic  $K^+/H^+$  antiporter was identified in a larval insect midgut vesicle preparation (Wieczorek et al., 1991) and its stoichiometry determined to be  $1K^+:2H^+$  (Azuma et al., 1995), but it had never been cloned.

### AgNHA1 leads to revised pH regulation paradigm

The abundant evidence that electroneutral  $Na^+/H^+$  exchangers (NHEs) are driven by  $Na^+$  gradients established by the  $Na^+/K^+$  P-ATPase (Orlowski and Grinstein, 2004) led to the dogma that virtually all  $Na^+/H^+$  exchange in metazoans is mediated by NHEs,

which provide the chief mechanism by which metabolic acid is extruded from animal cells. The second major class of electrophoretic NHAs was known mainly from studies on alkalophilic bacteria. However, phylogenetic analysis has shown that NHAs are found in all phyla for which genomes are available, from fungi through plants to mammals, including humans (Brett et al., 2005). Recently, the first metazoan NHA was cloned and localized in *An. gambiae* and named AgNHA1 (Rheault et al., 2007). These results, along with the immunolocalization data presented here (Table 1), suggest that the paradigm for pH regulation should be expanded as follows. Classical eukaryotic NHEs are electroneutral  $Na^+/H^+$  exchangers driven by  $Na^+$  gradients generated by  $Na^+/K^+$  P-ATPases whereas the new eukaryotic NHAs are expected to be electrophoretic  $Na^+/H^+$  antiporters driven by  $\Delta\Psi$ s that are usually generated by  $H^+$  V-ATPases.

### NHE<sub>V-NAT</sub>

The colocalization of  $H^+$  V-ATPase (red, Fig. 5C) and the  $Na^+$ :amino acid<sup>+/-</sup> symporter, AgNAT8 (red, Fig. 5F) on the apical membrane of the same cells in posterior midgut (model, Fig. 6) identifies a new type of NHE. The efflux of  $H^+$  from cells mediated by  $H^+$  V-ATPases linked to the influx of  $Na^+$  into cells *via* amino acid symporters (NATs) constitutes an overlooked  $Na^+/H^+$  exchange mechanism that has the same orientation as eukaryotic NHEs and which we will call NHE<sub>V-NAT</sub>.

### $H^+$ V-ATPase and $Na^+/K^+$ P-ATPases as plasma membrane energizers

Long after the discovery of the insect  $K^+$  pump (Anderson and Harvey, 1966; Gupta and Berridge, 1966; Maddrell, 1971; Ramsay,



1953) the  $\text{Na}^+/\text{K}^+$  P-ATPase (Skou, 1990; Ussing and Zerahn, 1951) was still widely regarded as the sole energizer of animal plasma membranes (Ussing, 1988). When the  $\text{K}^+$  pump-containing goblet cell apical membrane was isolated (Cioffi and Wolfersberger, 1983; Harvey et al., 1983a) and the  $\text{K}^+$  pump's molecular identity as a V-ATPase (Schweickl et al., 1989) coupled to a  $\text{K}^+/\text{2H}^+$  antiporter (Azuma et al., 1995; Wieczorek et al., 1991) was established, the pendulum swung so far in favor of V-ATPases that many insect workers still believe that all insect plasma membranes are energized by this membrane protein. The notion that both  $\text{H}^+$  V-ATPases and  $\text{Na}^+/\text{K}^+$  P-ATPases energize insect membranes was firmly established (Patrick et al., 2006) in larval and adult *Ae. aegypti* midgut. Figs 3 and 4 extend this finding to *An. gambiae* and establish beyond any reasonable doubt that both ATPases are abundant in insect plasma membranes, usually in the same cells. The default condition in insect membranes, as in the frog skin (Ehrenfeld and Klein, 1997) is that  $\text{H}^+$  V-ATPases are located on apical membranes and  $\text{Na}^+/\text{K}^+$  P-ATPases on basolateral membranes (simply basal membranes in insects). The caterpillar midgut represents an extreme deviation in which the  $\text{Na}^+/\text{K}^+$  P-ATPase is not detectable. The anterior midgut of mosquito larvae represents another deviation in that the polarity is reversed:  $\text{H}^+$  V-ATPases are located on basal membranes and  $\text{Na}^+/\text{K}^+$  P-ATPases are on apical membranes (Patrick et al., 2006; Zhuang et al., 1999).

#### NHAs localize with $\text{H}^+$ V-ATPases

Direct evidence that AgNHA1 is electrophoretic is a voltage-dependent inward current and  $\text{Na}^+$ -dependent cell acidification in AgNHA1-transfected *Xenopus* oocytes (L. B. Popova, D. Y. Boudko and W. R. Harvey, unpublished measurements). If AgNHA1 in mosquito midgut is driven by transmembrane voltages that are generated by  $\text{H}^+$  V-ATPases then AgNHA1 staining should colocalize with  $\text{H}^+$ -V-ATPase staining. Such a colocalization can be deduced by comparing the whole mounts labeled with AgNHA1 antibody (red, Fig. 3A) with those labeled by  $\text{H}^+$  V-ATPase antibody (red, Fig. 3B) all along the alimentary canal. The apparent colocalization of AgNHA1 and  $\text{H}^+$  V-ATPase can also be deduced in whole mounts of gastric caeca (Fig. 3C,D, respectively) and in paraffin sections of posterior midgut cells [Fig. 5C (see Rheault et al., 2007)]. Unexpectedly, AgNHA1 appears in a punctate pattern in anterior midgut (Fig. 3A), suggesting that it resides mainly in intracellular vesicles rather than in the apical membrane (see discussion below). The apical membranes in these enigmatic anterior midgut cells provide an exception to the V-ATPase–NHA colocalization rule in that AgNHA1 colocalizes with  $\text{Na}^+/\text{K}^+$  P-ATPase rather than with  $\text{H}^+$  V-ATPase.

#### Basal $\text{H}^+$ V-ATPase and apical $\text{Na}^+/\text{K}^+$ P-ATPase in mosquito anterior midgut

The mosquito anterior midgut evolved with the  $\text{H}^+$  V-ATPase on the basal membrane (Fig. 5B). This atypical placement is to be expected if the ATPase is to pump  $\text{H}^+$  out of the cells to the blood. A basal V-ATPase in anterior midgut was first observed by Moira Cioffi in *Ae. aegypti* larvae (Zhuang et al., 1999) and later confirmed (Patrick et al., 2006). This basal location of the electrogenic proton pump explains the large basal membrane hyperpolarization reported (Clark et al., 1999). Also atypical is the location of  $\text{Na}^+/\text{K}^+$  P-ATPase in the apical membrane where it would eject  $\text{Na}^+$  into the lumen and provide the counter ion for  $\text{CO}_3^{2-}$ . One might expect that AgNHA1 would also be located in the apical membrane in anterior midgut if it is to remove  $\text{H}^+$  from the lumen (Fig. 6) but the labeling was found in nearby cytoplasmic

vesicles, as discussed above. Nevertheless, under stressful conditions the vesicles could fuse with the apical membrane, insert the NHA and provide a pathway by which  $\text{H}^+$  from the lumen could replace that expelled to the blood by the  $\text{H}^+$  V-ATPase while complementing the apical  $\text{Na}^+/\text{K}^+$  P-ATPase in supplying  $\text{Na}^+$  to the lumen. The resulting  $2\text{Na}^+$  and  $\text{CO}_3^{2-}$  ( $\text{pK}_a$  10.2) would account for the high luminal pH in anterior midgut. A similar stress-induced fusion of apically located vesicles with the apical plasma membrane is well documented in the mammalian kidney and elsewhere (Brown and Breton, 1996). The 'stripping of  $\text{H}^+$  from  $\text{HCO}_3^-$  to produce  $\text{CO}_3^{2-}$  was first suggested (Dow, 1984) to explain the high pH in caterpillar anterior midgut.

#### NHE<sub>V-NAT</sub>, a novel, functional NHE in posterior midgut

Starting with the early Waterhouse paper on lepidopteran midgut alkalization (Waterhouse, 1949) and continuing with Dadd's report on high pH in mosquito larval midgut (Dadd, 1975) and Dow's remarkable paper on alkalization to a pH as high as 12 in the midgut of several caterpillars (Dow, 1984), the entire literature on pH in insects has been preoccupied with alkalization. Yet all of the reports summarized in the book by Clements (Clements, 1992) show that the pH does not remain high but starts to decline in central midgut and falls to values as low as 7.5 in posterior midgut. What is the mechanism for reversal of alkalization? The answer is surprisingly simple.  $\text{H}^+$  V-ATPase is localized along with ( $\text{K}^+$  or  $\text{Na}^+$ )-coupled amino acid symporters on the apical membranes in posterior midgut cells (Fig. 5C,D,F) (see also Boudko et al., 2005a; Meleshkevitch et al., 2006). It is widely accepted that V-ATPases hyperpolarize their resident membranes and the lumen-positive voltage drives electrophoretic ( $\text{K}^+$  or  $\text{Na}^+$ ):amino acid symport into the cells (Boudko et al., 2005b). The electrically coupled  $\text{H}^+$  V-ATPase and  $\text{Na}^+$ :amino acid<sup>+/−</sup> NAT together constitute an NHE (NHE<sub>V-NAT</sub>). The V-ATPase drives  $\text{H}^+$  out of the cells and the symporter drives  $\text{Na}^+$  into the cells. This functional  $\text{Na}^+/\text{H}^+$  exchanger does exactly what a classical NHE does. The difference is that this novel  $\text{Na}^+/\text{H}^+$  exchanger comprises two separate, electrically coupled transporters whereas the classical NHE is a single  $\text{Na}^+$ -gradient driven transporter. Since there are seven NATs in the *An. gambiae* genome (Boudko et al., 2005b), there are effectively nine NHEs: the two classical NHEs and seven NHE<sub>V-NAT</sub>s in this mosquito. One of the referees to this manuscript suggested that the NHE<sub>V-NAT</sub> concept 'will cause other ion transport physiologists to reassess current dogma in other ion transporting tissues of other invertebrates and vertebrates'. The question remaining is: what supplies the  $\text{H}^+$  to the V-ATPase and the  $\text{Na}^+$  to the amino acid symporter in posterior midgut? To answer this question one must consider amino acid uptake by the same cells.

#### AgNHA1 recycles $\text{H}^+$ from lumen to cell and $\text{Na}^+$ from cell to lumen

Amino acids are required for the ~1000-fold increase in larval mass during development and are the principal osmolytes in blood. Their massive uptake by  $\text{Na}^+$ -coupled symport from posterior midgut lumen would soon deplete  $\text{Na}^+$  there. Concomitantly,  $\text{H}^+$  entry via the membrane-energizing  $\text{H}^+$ -ATPase would soon decrease lumen pH to toxic levels. AgNHA1 and AgNAT8 are both localized on the apical membrane in posterior midgut cells (Fig. 5E), forming a NHE<sub>V-NAT</sub> that solves both problems. NHE<sub>V-NAT</sub> provides a pathway for recycling  $\text{Na}^+$  from cells to lumen and for recycling  $\text{H}^+$  from lumen to cells. Thus the localization of the  $\text{H}^+$  V-ATPase on the same membrane as the  $\text{Na}^+$ :amino acid<sup>+/−</sup> symporters and the ( $\text{Na}^+$  or  $\text{K}^+$ )/ $\text{nH}^+$  antiporter provides an integrated pathway for

pH homeostasis that can account for the measured decrease in pH to ~7.5 in posterior midgut lumen. Finally, although some of the amino acids absorbed across the apical membrane in posterior midgut are used for local protein synthesis and as energy substrates, most of them would be expected to leave the cells and enter the hemolymph. An AgNAT6 (B.A.O., D.Y.B. and W.R.H., unpublished data) on the basal membrane is positioned to mediate this exit (Fig. 6).

#### V<sub>1</sub>-ATPases in midgut epithelial cells are portosomes

The widespread occurrence of H<sup>+</sup> V-ATPase in larvae of *Ae. aegypti* (Patrick et al., 2006) and in *An. gambiae* reported here underscores the long-neglected fact that the cytoplasmic sector of the enzyme V<sub>1</sub>-ATPase can be seen in electron micrographs where they have been called 'portosomes' (Harvey, 1980; Harvey et al., 1981). They were first described as a 'coat of repeating subunits that stud the cytoplasmic surface of the apical plasma membranes in blowfly rectal papillae' (Gupta and Berridge, 1966); soon after, similar subunits were reported on the apical membranes of caterpillar midgut goblet cells (Anderson and Harvey, 1966). Portosomes are especially prominent in mosquitoes (Bradley et al., 1982; Clements, 1992) (Table 2). Based on thermodynamics and cytology, Harvey et al. (Harvey et al., 1983b) argued that portosomes are the equivalent of the F<sub>1</sub> sectors of ATP synthases (which can also function as F<sub>1</sub>-ATPases). V<sub>1</sub>-ATPases have been solubilized in pure form (Wieczorek et al., 1989) from isolated goblet cell apical membranes (Cioffi and Wolfersberger, 1983; Harvey, 1982) and shown beyond doubt to be portosomes (Grüber et al., 2000). Several examples of portosomes on apical and lateral membranes of rectal cells are described in the literature (e.g. Clements, 1992), but their identity as V<sub>1</sub>-ATPase sectors is rarely used to interpret results. An exception is the colocalization of portosomes with H<sup>+</sup> V-ATPase immunostaining and membrane hyperpolarization in the anterior midgut of *Ae. aegypti* larvae, which enabled Zhuang et al. (Zhuang et al., 1999) to demonstrate that H<sup>+</sup> V-ATPases are located on the basal membrane, a deduction that was confirmed (Patrick et al., 2006). In the present work, the colocalization of H<sup>+</sup> V-ATPase antibody staining (Fig. 5A,B) with the published location of portosomes (e.g. Clements, 1992; Volkmann and Peters, 1989) (Table 2) enables one to deduce that the resident membranes are hyperpolarized and can furnish the energy required to drive ion-coupled transporters, such as NATs. This deduction helps to analyze the cell biology of the rectum as discussed below.

#### A two part rectum in *An. gambiae* larvae is revealed by immunolocalization

The *An. gambiae* larval rectum was labeled differentially with the Na<sup>+</sup>/K<sup>+</sup>-ATPase and H<sup>+</sup> V-ATPase antibodies, implying that it has two parts, since these enzymes energize biomembranes in quite different ways. Although both parts were labeled by H<sup>+</sup> V-ATPase and AgNAT8 antibody (data not shown), Na<sup>+</sup>/K<sup>+</sup> P-ATPase was not detected in dorsal anterior rectum (Fig. 4). Instead, carbonic anhydrase (AgCA9) was found in the DAR cells but not detected in posterior rectum (Smith et al., 2007). Labeling by antibodies to AgNHA1 and nutrient amino acid transporters (NATs) was prominent. The H<sup>+</sup> V-ATPase hyperpolarizes the apical membrane and the Na<sup>+</sup> coupled NATs use the voltage to drive Na<sup>+</sup> and amino acids into the cells. Meanwhile the voltage is also used by the AgNHA1 to recycle the H<sup>+</sup> back into the cells and the Na<sup>+</sup> back into the lumen (Fig. 6). The basal membranes of posterior rectum cells are labeled with antibody to Na<sup>+</sup>/K<sup>+</sup> P-

ATPase which mediates Na<sup>+</sup> reabsorption to the blood. This reabsorption enables mosquito larvae, living in fresh water with a [Na<sup>+</sup>] <0.0001 mol l<sup>-1</sup>, to conserve this ion, which is required for anterior midgut alkalinization, amino acid uptake and nerve function

In summary, anterior dorsal rectum appears to be the site for bicarbonate secretion and chloride absorption in fresh water larvae, similar to that demonstrated some time ago in euryhaline insects (Strange, 1982; Strange and Phillips, 1984; Strange and Phillips, 1985; Strange et al., 1984) whereas posterior rectum appears to provide for Na<sup>+</sup> conservation. Overall, the rectum provides a mechanism by which Na<sup>+</sup>, K<sup>+</sup>, Cl<sup>-</sup> and amino acids are absorbed into the blood before carbonic acid and the rest of the luminal contents are expelled to the environment.

The carbonic anhydrase in the rectal cells (Smith et al., 2007) implies that metabolic CO<sub>2</sub> is hydrated to HCO<sub>3</sub><sup>-</sup> which is secreted to the lumen, *via* a postulated (Stobbart, 1971) but not identified Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger (AE). Meanwhile, the apical H<sup>+</sup> V-ATPase supplies H<sup>+</sup> to the lumen to yield H<sub>2</sub>CO<sub>3</sub>, the weak carbonic acid. If this pathway is also present in *Ae. aegypti* larvae, it would account for the acidity of the rectal lumen reported (Clark et al., 2007). Other reports confirmed that *Ae. aegypti* larvae alkalinize the medium under certain experimental conditions (del Pilar Corena et al., 2004; Stobbart, 1971). Stobbart argued that the likely bases are NH<sub>4</sub>OH and KHCO<sub>3</sub>. Stobbart also detected Cl<sup>-</sup> uptake from KCl, about half of which was by exchange for HCO<sub>3</sub><sup>-</sup>. Moreover, he also calculated that about half of the Na<sup>+</sup> uptake was by exchange for H<sup>+</sup>. Thus, as early as 1971, Stobbart had predicted the presence of Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchangers (NHEs and AEs) in larval mosquito rectum. del Pilar Corena et al. added to this picture by showing that carbonic anhydrase inhibitors also block excretion of bases (del Pilar Corena et al., 2004).

This labeling pattern implies a two-part functional rectum that is analogous to the two-part rectum of osmoregulating, euryhaline mosquito larvae, such as *Ae. campestris* and *Ochlerotatus taeniorhynchus* (Grueber and Bradley, 1994). The presence of carbonic anhydrase suggests that the function of the dorsal anterior rectal cells is to replace Cl<sup>-</sup> in the lumen by HCO<sub>3</sub><sup>-</sup> from cell metabolism *via* a postulated, but as yet unidentified, electrophoretic anion exchanger that is driven by the voltage from the H<sup>+</sup> V-ATPase. The posterior rectum appears to be the site of the final absorption of Na<sup>+</sup> from the lumen.

#### CONCLUSIONS

(1) The localizations of four components of the cationic pathway reported here, along with the recent report of the phylogeny, cloning and localization of the first eukaryotic NHA (Rheault et al., 2007) lead to an expansion of the paradigm for pH regulation in transporting epithelia: Na<sup>+</sup>/K<sup>+</sup> P-ATPases generate Na<sup>+</sup> gradients that drive electroneutral Na<sup>+</sup>/H<sup>+</sup> exchangers by NHEs whereas H<sup>+</sup> V-ATPases generate voltage gradients that drive electrophoretic Na<sup>+</sup>/H<sup>+</sup> antiporters by NHAs. A corollary to this paradigm is that NHAs will usually be colocalized with H<sup>+</sup> V-ATPases [for an exception see Beyenbach's (Beyenbach, 2001) discussion of electrical coupling between apical and basal membranes]. (2) Co-existence of H<sup>+</sup> V-ATPases and NATs on the same membranes constitutes a new functional NHE (NHE<sub>V-NAT</sub>). (3) Labeling with antibody to H<sup>+</sup> V-ATPase of membranes previously reported to contain portosomes provides new insight into the function of studded membranes – they will contain H<sup>+</sup> V-ATPase, which will hyperpolarize them and energize resident transporters. (4) Co-existence of H<sup>+</sup> V-ATPases,

NHAs and NATs on the same membranes provides local recycling mechanisms for both H<sup>+</sup> and Na<sup>+</sup> (Fig. 6).

### LIST OF ABBREVIATIONS

AgNHA	<i>Anopheles gambiae</i> Na <sup>+</sup> /H <sup>+</sup> antiporter
AgNHE	<i>Anopheles gambiae</i> Na <sup>+</sup> /H <sup>+</sup> exchanger
DAR cells	dorsal anterior rectum cells
NAT	nutrient amino acid transporter (Na <sup>+</sup> or K <sup>+</sup> )-coupled amino acid transporter
NHA	Na <sup>+</sup> /H <sup>+</sup> antiporter
NHE <sub>V-NAT</sub>	functional Na <sup>+</sup> /H <sup>+</sup> exchanger (H <sup>+</sup> V-ATPase electrically coupled to NAT)
NHE	Na <sup>+</sup> /H <sup>+</sup> exchanger

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### REFERENCES

- Abramoff, M. D., Magelhaes, P. J. and Ram, S. J. (2004). Image processing with ImageJ. *Biophotonics Int.* **11**, 36-42.
- Anderson, E. and Harvey, W. R. (1966). Active transport by the *Cecropia* midgut. II. Fine structure of the midgut epithelium. *J. Cell Biol.* **31**, 107-137.
- Arkin, I. T., Xu, H., Jensen, M. O., Arbely, E., Bennett, E. R., Bowers, K. J., Chow, E., Dror, R. O., Eastwood, M. P., Flitman-Tene, R. et al. (2007). Mechanism of Na<sup>+</sup>/H<sup>+</sup> antiporting. *Science* **317**, 799-803.
- Azuma, M., Harvey, W. R. and Wiczorek, H. (1995). Stoichiometry of K<sup>+</sup>/H<sup>+</sup> antiporter helps to explain extracellular pH 11 in a model epithelium. *FEBS Lett.* **361**, 153-156.
- Beyenbach, K. W. (2001). Energizing epithelial transport with the vacuolar H<sup>(+)</sup>-ATPase. *News Physiol. Sci.* **16**, 145-151.
- Boudko, D. Y., Moroz, L. L., Harvey, W. R. and Linser, P. J. (2001a). Alkalinization by chloride/bicarbonate pathway in larval mosquito midgut. *Proc. Natl. Acad. Sci. USA* **98**, 15354-15359.
- Boudko, D. Y., Moroz, L. L., Linser, P. J., Trimarchi, J. R., Smith, P. J. S. and Harvey, W. R. (2001b). In situ analysis of pH gradients in mosquito larvae using noninvasive, self-referencing, pH-sensitive microelectrodes. *J. Exp. Biol.* **204**, 691-699.
- Boudko, D. Y., Kohn, A. B., Meleshkevitch, E. A., Dasher, M. K., Seron, T. J., Stevens, B. R. and Harvey, W. R. (2005a). Ancestry and progeny of nutrient amino acid transporters. *Proc. Natl. Acad. Sci. USA* **102**, 1360-1365.
- Boudko, D. Y., Stevens, B. R., Donly, B. C. and Harvey, W. R. (2005b). Nutrient amino acid and neurotransmitter transporters. In *Comprehensive Molecular Insect Science*. Vol. 4 (ed. K. Latrou, S. S. Gill and L. I. Gilbert), pp. 255-309. Amsterdam: Elsevier.
- Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.
- Bradley, T. J., Stuart, A. M. and Satir, P. (1982). The ultrastructure of the larval malpighian tubules of a saline-water mosquito. *Tissue Cell* **14**, 759-773.
- Brett, C. L., Donowitz, M. and Rao, R. (2005). Evolutionary origins of eukaryotic sodium/proton exchangers. *Am. J. Physiol.* **288**, C223-C239.
- Brown, D. and Breton, S. (1996). Mitochondria-rich, proton-secreting epithelial cells. *J. Exp. Biol.* **199**, 2345-2358.
- Cioffi, M. (1984). Comparative ultrastructure of arthropod transporting epithelia. *Am. Zool.* **24**, 139-156.
- Cioffi, M. and Wolfersberger, M. G. (1983). Isolation of separate apical, lateral and basal plasma membrane from cells of an insect epithelium. A procedure based on tissue organization and ultrastructure. *Tissue Cell* **15**, 781-803.
- Clark, T. M., Koch, A. and Moffett, D. F. (1999). The anterior and posterior 'stomach' regions of larval *Aedes aegypti* midgut: regional specialization of ion transport and stimulation by 5-hydroxytryptamine. *J. Exp. Biol.* **202**, 247-252.
- Clark, T. M., Vieira, M. A. L., Huegel, K. L., Flury, D., Carper, M. (2007). Strategies for regulation of hemolymph pH in acidic and alkaline water by the larval mosquito *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Exp. Biol.* **210**, 4359-4367.
- Clements, A. N. (1992). *The Biology of Mosquitoes*. London: Chapman & Hall.
- Corena, M. P., Seron, T. J., Lehman, H. K., Ochrietor, J. D., Kohn, A., Tu, C. and Linser, P. J. (2002). Carbonic anhydrase in the midgut of larval *Aedes aegypti*: cloning, localization and inhibition. *J. Exp. Biol.* **205**, 591-602.
- Dadd, R. H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect Physiol.* **21**, 1847-1853.
- del Pilar Corena, M., Fiedler, M. M., VanEkeris, L., Tu, C., Silverman, D. N. and Linser, P. J. (2004). Alkalinization of larval mosquito midgut and the role of carbonic anhydrase in different species of mosquitoes. *Comp. Biochem. Physiol.* **137C**, 207-225.
- Dow, J. A. T. (1984). Extremely high pH in biological systems: a model for carbonate transport. *Am. J. Physiol.* **246**, R633-R636.
- Ehrenfeld, J. and Klein, U. (1997). The key role of the H<sup>+</sup> V-ATPase in acid-base balance and Na<sup>+</sup> transport processes in frog skin. *J. Exp. Biol.* **200**, 247-256.
- Filippova, M., Ross, L. S. and Gill, S. S. (1998). Cloning of the V-ATPase B subunit cDNA from *Culex quinquefasciatus* and expression of the B and C subunits in mosquitoes. *Insect Mol. Biol.* **7**, 223-232.
- Grüber, G., Radermacher, M., Ruiz, T., Godovac-Zimmermann, J., Canas, B., Kleine-Kohlbrecher, D., Huss, M., Harvey, W. R. and Wiczorek, H. (2000). Three-dimensional structure and subunit topology of the V(1) ATPase from *Manduca sexta* midgut. *Biochemistry* **39**, 8609-8616.
- Grueber, W. B. and Bradley, T. J. (1994). The evolution of increased salinity tolerance in larvae of *Aedes* mosquitoes: a phylogenetic analysis. *Physiol. Zool.* **67**, 566-579.
- Gupta, B. L. and Berridge, M. J. (1966). A coat of repeating subunits on the cytoplasmic surface of the plasma membrane in the rectal papillae of the blowfly, *Calliphora erythrocephala* (Meig.), studied in situ by electron microscopy. *J. Cell Biol.* **29**, 376-382.
- Harvey, W. R. (1980). Water and ions in the gut. In *Insect Biology in the Future* (ed. M. Locke and D. S. Smith), pp. 105-124. New York: Academic Press.
- Harvey, W. R. (1982). Membrane physiology of insects. In *Membrane Physiology of Invertebrates* (ed. R. B. Podesta), pp. 495-566. New York, Basel: Marcel Dekker.
- Harvey, W. R. and Wiczorek, H. (1997). Animal plasma membrane energization by chemiosmotic H<sup>+</sup> V-ATPases. *J. Exp. Biol.* **200**, 203-216.
- Harvey, W. R., Cioffi, M. and Wolfersberger, M. G. (1981). Portosomes as coupling factors in active ion transport and oxidative phosphorylation. *Am. Zool.* **21**, 775-791.
- Harvey, W. R., Cioffi, M., Dow, J. A. and Wolfersberger, M. G. (1983a). Potassium ion transport ATPase in insect epithelia. *J. Exp. Biol.* **106**, 91-117.
- Harvey, W. R., Cioffi, M. and Wolfersberger, M. G. (1983b). Chemiosmotic potassium ion pump of insect epithelia. *Am. J. Physiol.* **244**, R163-R175.
- Hunte, C., Screpanti, E., Venturi, M., Rimon, A., Padan, E. and Michel, H. (2005). Structure of a Na<sup>+</sup>/H<sup>+</sup> antiporter and insights into mechanism of action and regulation by pH. *Nature* **435**, 1197-1202.
- Maddrell, S. H. P. (1971). Fluid secretion by the Malpighian tubules of insects. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **262**, 197-207.
- Meleshkevitch, E. A., Assis-Nascimento, P., Popova, L. B., Miller, M. M., Kohn, A. B., Phung, E. N., Mandal, A., Harvey, W. R. and Boudko, D. Y. (2006). Molecular characterization of the first aromatic nutrient transporter from the sodium neurotransmitter symporter family. *J. Exp. Biol.* **209**, 3183-3198.
- Orlowski, J. and Grinstein, S. (1997). Na<sup>+</sup>/H<sup>+</sup> exchangers of mammalian cells. *J. Biol. Chem.* **272**, 22373-22376.
- Orlowski, J. and Grinstein, S. (2004). Diversity of the mammalian sodium/proton exchanger SLC9 gene family. *Pflügers Arch.* **447**, 549-565.
- Padan, E., Bibi, E., Ito, M. and Krulwich, T. A. (2005). Alkaline pH homeostasis in bacteria: new insights. *Biochim. Biophys. Acta* **1717**, 67-88.
- Patrick, M. L., Aimanova, K., Sanders, H. R. and Gill, S. S. (2006). P-type Na<sup>+</sup>/K<sup>+</sup>-ATPase and V-type H<sup>+</sup>-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. *J. Exp. Biol.* **209**, 4638-4651.
- Ramsay, J. A. (1950). Osmotic regulation in mosquito larvae. *J. Exp. Biol.* **27**, 145-157.
- Ramsay, J. A. (1953). Active transport of potassium by the malpighian tubules of insects. *J. Exp. Biol.* **30**, 358-369.
- Rheault, M. R., Okech, B. A., Keen, S. B., Miller, M. M., Meleshkevitch, E. A., Linser, P. J., Boudko, D. Y. and Harvey, W. R. (2007). Molecular cloning, phylogeny and localization of AgNHA1: the first Na<sup>+</sup>/H<sup>+</sup> antiporter (NHA) from a metazoan, *Anopheles gambiae*. *J. Exp. Biol.* **210**, 3848-3861.
- Schweikl, H., Klein, U., Schindlbeck, M. and Wiczorek, H. (1989). A vacuolar-type ATPase, partially purified from potassium transporting plasma membranes of tobacco hornworm midgut. *J. Biol. Chem.* **264**, 11136-11142.
- Skou, J. C. (1990). The energy coupled exchange of Na<sup>+</sup> for K<sup>+</sup> across the cell membrane - the Na<sup>+</sup>, K<sup>+</sup>-pump. *FEBS Lett.* **268**, 314-324.
- Smith, K. E., VanEkeris, L. A. and Linser, P. J. (2007). Cloning and characterization of AgCA9, a novel alpha-carbonic anhydrase from *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) larvae. *J. Exp. Biol.* **210**, 3919-3930.
- Stobart, R. H. (1971). Evidence for Na<sup>+</sup>/H<sup>+</sup> and Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanges during independent sodium and chloride uptake by the larva of the mosquito *Aedes aegypti* (L.). *J. Exp. Biol.* **54**, 19-27.
- Strange, K. and Phillips, J. E. (1984). Mechanisms of CO<sub>2</sub> transport in rectal salt gland of *Aedes*. I. Ionic requirements of CO<sub>2</sub> secretion. *Am. J. Physiol.* **246**, R727-R734.
- Strange, K. and Phillips, J. E. (1985). Cellular mechanism of HCO<sub>3</sub><sup>-</sup> and Cl<sup>-</sup> transport in insect salt gland. *J. Membr. Biol.* **83**, 25-37.
- Strange, K., Phillips, J. E. and Quamme, G. A. (1982). Active HCO<sub>3</sub><sup>-</sup> secretion in the rectal salt gland of a mosquito larva inhabiting NaHCO<sub>3</sub>-CO<sub>3</sub> lakes. *J. Exp. Biol.* **101**, 171-186.
- Strange, K., Phillips, J. E. and Quamme, G. A. (1984). Mechanisms of CO<sub>2</sub> transport in rectal salt gland of *Aedes*. II. Site of Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchange. *Am. J. Physiol.* **246**, R735-R740.
- Turbeck, B. O., Nedergaard, S. and Kruse, H. (1968). An anion-stimulated adenosine triphosphatase from the potassium-transporting midgut of the larva of *Hyalophora cecropia*. *Biochim. Biophys. Acta* **163**, 354-361.
- Ussing, H. H. (1988). The development of the concept of active transport. *Prog. Clin. Biol. Res.* **268B**, 3-16.
- Ussing, H. H. and Zerahn, K. (1951). Active transport of sodium as the source of electric current in the short-circuited isolated frog skin. *Acta Physiol. Scand.* **23**, 110-127.
- Volkman, A. and Peters, W. (1989). Investigations on the midgut caeca of mosquito larvae. I. Fine structure. *Tissue Cell* **21**, 243-251.



- Waterhouse, D. F.** (1949). The hydrogen ion concentration in the alimentary canal of larval and adult Lepidoptera. *Aust. J. Sci. Res. B* **428**, 437.
- Wieczorek, H., Weerth, S., Schindlbeck, M. and Klein, U.** (1989). A vacuolar-type proton pump in a vesicle fraction enriched with potassium transporting plasma membranes from tobacco hornworm midgut. *J. Biol. Chem.* **264**, 11143-11148.
- Wieczorek, H., Putzenlechner, M., Zeiske, W. and Klein, U.** (1991). A vacuolar-type proton pump energizes K<sup>+</sup>/H<sup>+</sup> antiport in an animal plasma membrane. *J. Biol. Chem.* **266**, 15340-15347.
- Wieczorek, H., Brown, D., Grinstein, S., Ehrenfeld, J. and Harvey, W. R.** (1999). Animal plasma membrane energization by proton-motive V-ATPases. *BioEssays* **21**, 637-648.
- Wigglesworth, V.** (1972). *Principles of Insect Physiology*. London: Chapman & Hall.
- Zhuang, Z., Linser, P. J. and Harvey, W. R.** (1999). Antibody to H<sup>+</sup> V-ATPase subunit E colocalizes with portosomes in alkaline larval midgut of a freshwater mosquito (*Aedes aegypti*). *J. Exp. Biol.* **202**, 2449-2460.