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Slc4-like anion transporters of the larval mosquito alimentary canal

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Abstract

Mosquito larvae exhibit luminal pH extremes along the axial length of their alimentary canal that range from very alkaline (pH > 10) in the anterior midgut to slightly acid in the hindgut. The principal buffer in the system is thought to be bicarbonate and/or carbonate, because the lumen is known to contain high levels of bicarbonate/carbonate and is surrounded by various epithelial cell types which express a variety of carbonic anhydrases. However, the precise mechanisms responsible for the transport of bicarbonate/carbonate into and out of the lumen are unclear. In the present study, we test the hypothesis that SLC4-like anion transporters play a role in bicarbonate/carbonate accumulation in the larval mosquito alimentary canal. Molecular, physiological and immunohistochemical characterizations of Slc4-like transporters in the gut of larval mosquitoes (*Aedes aegypti* and *Anopheles gambiae*) demonstrate the presence of both a Na⁺-independent chloride/bicarbonate anion exchanger (AE) as well as a Na⁺-dependent anion exchanger (NDAE). Notably, immunolocalization experiments in Malpighian tubules show that the two proteins can be located in the same tissue, but to different cell types. Immunolabeling experiments in the gastric caecae show that the two proteins can be found in the same cells, but on opposite sides (basal vs. apical). In summary, our results indicate that the alimentary canal of larval mosquitoes exhibits robust expression of two SLC4-like transporters in locations that are consistent with a role in the regulation of luminal pH. The precise physiological contributions of each transporter remain to be determined.

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Specific contributions of authors: PJJ performed all immunochemical analyses and wrote the majority of the manuscript; MNO cloned AgNDAE1 cDNA variant 2 and designed the antibody probes for this protein; MFR and TH cloned AgNDAE1 cDNA variants 3 and 4 and performed oocyte expression experiments and physiological analyses; TJS cloned AgNDAE1 variant 1; KES cloned AgAE2; PMP provided antibody probes to AaAE1 and contributed to manuscript preparation; MFR performed data analyses on oocyte physiological experiments and contributed to manuscript preparation and editing. The current affiliation for TJS is Flagler College, St. Augustine Florida, for KES is the Mayo Clinic, Jacksonville, Florida and for MNO is Oxitech Limited, Abington UK.

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Keywords

Anion exchangers; mosquito larvae; alimentary canal; Malpighian tubule; Gastric caeca

Introduction

Mosquitoes are considered the most dangerous animals on earth by the World Health Organization (WHO). The bite of the female mosquito can transmit such deadly and debilitating human diseases as Malaria, Yellow Fever, Dengue Fever, West Nile Virus disease and others (The WHO Report, 1996). Millions of people die as a result of mosquito bites each year and countless billions more suffer a range of diseases which impact a healthy and productive life. Mankind has been struggling to control mosquito populations worldwide for most of our history either due to their extreme nuisance potential or more recently as a focus of disease mitigation. Control of populations of any organism is always derivative of some understanding of that organism's biology. Despite many decades of research, much remains to be learned about fundamental mosquito biology. The existing commonly used chemical agents for mosquito control target limited aspects of a mosquito's biology, such as a few specific targets in the nervous system, juvenile to adult development and larval gut cells. One very disturbing reality is that most of the registered control agents used worldwide are losing efficacy as populations of mosquitoes are selected for resistance by the heavy usage of those control agents. As is true in the case of antibiotics used to treat bacterial infections, focused use of specific agents eventually makes those agents useless as the bacterial population evolves under the selective pressure. Also as it is true with antibiotic development, the realm of mosquito control is always in need of new strategies and agents to use for control. Arguably the best place to look for new targets for the development of novel and yet environmentally safe control agents is into the fundamental biology of the mosquito itself.

A particular biological system of the mosquito that is poorly understood and yet highly specialized is the molecular physiology of the handling of the by-products of aerobic metabolism and catabolism, CO_2 , HCO_3^- , and CO_3^{2-} . In adult female mosquitoes, the event of a blood meal not only provides vast resources for the support of egg development, it also introduces fluids many times greater in mass than the mosquito itself. Those fluids are a concentrated source of CO_2 and HCO_3^- both in the serum and within the red blood cells. As the blood meal is digested, the excess CO_2 and metabolites must be disposed of without driving the mosquito's own ionic balance out of a life sustaining range.

Larval mosquitoes also have characteristics that indicate the need for specialized regulation of CO_2 and its other forms. Like a few other larval insects, mosquito larvae use an alkaline environment in the lumen of the alimentary canal to initiate digestion (Dadd, 1975; Zhuang et al., 1999; Corena et al., 2004). In the most alkaline regions of the larval gut, the pH can exceed 11 and in some caterpillars it can even reach 12 (Gringorten et al., 1993). In the living mosquito larva, $\text{HCO}_3^-/\text{CO}_3^{2-}$ is highly concentrated in the lumen (Boudko et al., 2001). The gut contains high levels of a variety of carbonic anhydrases in regionalized patterns of expression (Corena et al., 2002; Seron et al., 2004; Smith et al., 2007; Clark et al., 2007; Smith et al., 2008; Linser et al., 2009). An assumption has been made that bicarbonate transporters of the Slc4 type are likely to be expressed in the larval mosquito gut such that they might influence pH regulation. But to date, no analyses of the expression and specific localization of such transporters has been reported for mosquito larvae. In 2000 and 2001, Romero et al published the first description of an insect Slc4 homologue, a sodium-dependent anion exchanger (NDAE1) from *Drosophila melanogaster* (Romero et al., 2000; Sciortino et al., 2001). Recently, the first characterization of an Slc4-like $\text{Cl}^-/\text{HCO}_3^-$ anion

exchanger (AE) from a mosquito was published for the adult Yellow Fever mosquito *Aedes aegypti* (Aa) (Piermarini et al., 2010). Also, phylogenetic analyses of SLC4-like gene sequences in Aa and in *Anopheles gambiae* (Ag) were reported in 2009 (Linser et al., 2009). Microarray analyses demonstrate that transcripts of the members of this gene family in Ag larvae are expressed in tissue- and cell-type specific patterns in the alimentary canal (Neira-Oviedo et al., 2008, 2009; *ibid*). In the present study, we report the cloning and characterization of an NDAE1-like anion exchanger (AE) from larval Ag cDNA (AgNDAE1). Physiological evaluation of the encoded protein expressed heterologously in *Xenopus* oocytes shows it to in fact function as a Na⁺-driven Cl⁻/HCO₃⁻ transporter. Furthermore, we utilize immunochemical methods to compare and contrast the distribution of NDAE1 to AE1; the later has been described previously for adult Aa Malpighian tubules (AaAE1, Piermarini et al., 2010).

Materials & Methods

Animals

Fertilized eggs of *Anopheles gambiae* ss (Ag) were purchased from the CDC-Atlanta. The eggs were hatched in deionized water and maintained at 28 C° in an incubator with a 12/12 light dark cycle. Larvae were fed ground fish flakes every other day. Fertilized eggs of *Aedes aegypti* (Aa) were obtained from the USDA laboratory in Gainesville Florida. Eggs were hatched in deionized water and maintained as described above except that feeding was with a mixture of bakers yeast and liver powder also every other day. Adult Ag were obtained at the Johns Hopkins Bloomberg School of Public Health and injected with fixative prior to shipment to Florida. Aa were grown to adult emergence and maintained in the lab on 30% sucrose/water for several days prior to harvest and tissue dissection or chemical fixation. *X. laevis* were housed and cared for in accordance and approval of the Institutional Care and Use Committees of the Mayo Clinic.

cDNA cloning of AgNDAE1 variants

Standard cDNA cloning strategies were used to produce the four different variants of AgNDAE1 mRNA presented herein. cDNA collections were prepared from early 4th instar *Anopheles gambiae* ss (Ag) larvae as previously described (Smith et al., 2007). PCR primer sets were designed based on annotated gene sequence data and available ESTs. Full length transcripts were generated by RACE and sub-cloned into PCR4-TOPO (In Vitrogen, Carlsbad CA) as described (*ibid*). All clones were sequenced via Big Dye Terminator Sequencing (PE Biosystems, Foster CA, USA).

Oocyte expression and physiological characterization of AgNDAE1

The coding sequence for both *Anopheles gambiae* NDAE1 (AgNDAE1) and *Drosophila* NDAE1 (dNDAE1) were sub-cloned into the *Xenopus* oocyte expression vector pGEMHE (Liman et al., 1992). AgNDAE1 and dNDAE1 cRNA were synthesized *in vitro* using the T7 mMessage Machine kit (Ambion, Austin, TX). Stage V/VI oocytes were isolated by limited collagenase digestion (Romero et al., 1998). Oocytes were injected with 25 ng cRNA in 50 nl (0.5 µg/µL) water or 50 nl RNase-free water (control). Oocytes were studied 3–7 days after injection in a perfusion chamber monitoring pH_i and V_m as previously described (Romero et al., 1997; Romero et al., 2000; Kato et al., 2009). Oocytes were perfused with ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂ and 5 mM HEPES, pH 7.5, 195±5 mOsm). In CO₂/HCO₃⁻ equilibrated solutions, 33 mM NaHCO₃ replaced 33 mM NaCl and pH 7.5 was maintained by continuous bubbling with 5% CO₂/95% O₂ (Romero et al., 2000). Recordings shown in Fig. 2 are each representative of results from 4 to 6 oocytes.

Since changes in intracellular $[Cl^-]$ were not obvious in AgNDAE1-expressing oocytes in comparison to dNDAE1, we monitored intracellular $[I^-]$ using a halide-sensitive microelectrode (Bellemer et al., 2011). PMID 21427702>. This approach greatly increases the signal-to-noise ratio for detecting halide transport as free $[I^-]$ is typically $<1 \mu M$ in most vertebrate cells. The chloride ionophore will also respond to I^- , Br^- and SCN^- , allowing these electrodes to be used as halide-sensitive microelectrodes. However, we calibrated the [iodide] in solution as previously (Bellemer, 2011): for each electrode $[I^-]$ was varied between $100 \mu M$ and $10 \mu M$ (NaI) in $40 mM$ NaCl solution. This approach allowed us to determine an offset of $100 \mu M$ I^- in constant to $[Cl^-]$ background. The I^- -specific electrode slope was then determined between $100 \mu M$ and $10 \mu M$ NaI and this value used after the experiment to convert the data file to real $[I^-]$.

Antibodies

Rabbit polyclonal antibodies to Aa AE1 (AaAE1) have been described (Piermarini et al., 2010). Chicken polyclonal antibodies to Ag CA9 (carbonic anhydrase-9) have been described (Smith et al., 2007). Mouse monoclonal antibody (as hybridoma tissue culture supernatant) to the alpha-5 subunit of chicken Na^+/K^+ -ATPase originally generated by D. Fambrough was obtained from the Developmental Studies Hybridoma Bank, Iowa City IO, USA (<http://dshb.biology.uiowa.edu>) and used as previously described (Patrick et al., 2006; Smith et al., 2007). Polyclonal guinea pig antiserum to V-ATPase was generously provided by H. Wiczorek of the University of Osnabrück, Osnabrück Germany. To produce rabbit antisera to AgNDAE1, 100 amino acids from the computer-generated translation of the amino terminal cytoplasmic domain were assessed for immunogenic potential and then chosen for Genomic Antibody Technology (GAT) (see Figure 1 for sequence). Strategic Diagnostics Inc., Newark NJ, USA was contracted to produce two rabbit antisera using their proprietary DNA Antigen technology. Two rabbits were prescreened and chosen (Q3701 and Q3725) and the resultant polyclonal antisera provided identical results as reported herein.

Immunochemical analyses

SDS-PAGE and immunoblotting analyses were by standard methods as previously reported (e.g. Smith et al., 2007). Immunohistochemical analyses were performed on dissected larval and adult mosquito alimentary canal tissues by first injecting living animals with 2% p-formaldehyde in $0.1M$ Na-cacodylate buffer followed immediately by dissection in the same primary fixative solution. Tissues were stored (cold) in the primary fixative for a minimum of 2 hours and up to 5 days. Then the tissues were washed extensively in Tris-buffered saline (TBS) followed by pre-incubation medium (0.1% TritonX100, 1% normal goat serum, 1% bovine serum albumin in TBS [PreInc]) for a minimum of 1 hour. Then primary antibodies were applied diluted in PreInc (all polyclonal antibodies were diluted 1:1000 and the mouse monoclonal hybridoma supernatant 1:10) overnight at $4^\circ C$. Following extensive washing in PreInc the following day, appropriate secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove PA, USA) diluted 1:500 in PreInc were applied for 2 hours at $37^\circ C$. Tissues were again washed extensively in PreInc followed by TBS containing the Hoechst DNA stain DAPI at $300 nM$ for 5 minutes. After subsequent washing, tissues were mounted on glass slides using 60% glycerol/TBS containing $1mg/ml$ p-phenylenediamine (to retard fading). For histological sections of mosquito larvae, early 4th instar larvae were injected with primary fixative as above and then immersed in the fixative solution for 12 hours to several days at $4^\circ C$. Then the larvae were moved to fresh, ice-cold Carnoy's (secondary fixative) and then incubated over night at $4^\circ C$. Fixed larvae were taken through alcohol and methyl salicylate transition solvents and embedded in paraffin as described (e.g. Smith et al., 2007). Histological sections were cut at 6–12 microns and mounted on SuperFrost charged slides (Thermo/Fisher USA). After

rehydration, slides with sections were placed in 0.1 M Na-citrate pH 6.0 and heated in an autoclave to sterilization temperature for 15 minutes as an “antigen recovery” technique. Then slides with sections were transferred to TBS followed by PreInc. Antibody application to the histological sectioned material was essentially as described above.

For immunohistochemical staining of *Xenopus* oocytes, the oocytes were quick-frozen in OCT compound and then cryosectioned and the sections mounted on SuperFrost charged slides and stored at -80°C . Prior to immunostaining, the sections on slides were immersed in 2% p-formaldehyde in cacodylate buffer (see above) for 30 minutes at room temperature and then washed extensively with TBS followed by PreInc. Immunostaining of the oocyte sections was performed as described above for other histological sections. All stained materials were viewed with a Leica TCS SP5 laser scanning confocal microscope and digital images were gathered and then assembled into figures using CorelDRAW12 graphics suite.

Results

In the present study, we use the nomenclature scheme of Romero et al. (2000) and Piermarini et al. (2010) for identifying the mosquito Slc4 members. That is, the Na^{+} -independent chloride bicarbonate anion exchangers are designated as ‘AE’, and the Na^{+} -dependent anion exchangers are designated as ‘NDAE’. The numbers following an abbreviation (e.g., AE1) are arbitrary and do not reflect a similarity to a particular mammalian ortholog. This nomenclature scheme updates that of Linser et al. 2009 in which all mosquito Slc4 members were designated as ‘AE’. Table 1 reconciles the differences in nomenclature between Linser et al. 2009 and the present study.

Figure 1 shows an alignment of amino acid sequences for the putative Slc4 gene family of *Ag* and *Aa* according to the most recent annotations of the respective genomes (Holt et al., 2002; Vishvanath et al., 2007). Current annotation identifies two such genes in *Aa* and three in *Ag* (Linser et al., 2009; Piermarini et al. 2010). Two of the *Ag* genes and one of the *Aa* genes are most similar to the Na^{+} -independent chloride-bicarbonate (anion) exchangers (herein referred to as *Ag*AE1 and *Ag*AE2 and *Aa*AE1). Both *Ag* and *Aa* possess one gene each that is most similar to *Drosophila* NDAE1 (Romero et al., 2000; referred to herein as *Ag*NDAE1 and *Aa*NDAE1). The foci of this manuscript are the two proteins/transcripts/genes referred to herein as NDAE1 (Na^{+} dependent) and AE1 (Na^{+} independent). Although the *Ag*AE2 mRNA sequence has been cloned as a cDNA and deposited into GenBank (accession number EU068741) its expression shows little evidence of differential expression in alimentary canal tissues in *Ag* larvae and hence is not further pursued in this report (Neira-Oviedo et al., 2008, 2009, Linser et al., 2009). In contrast, both *Ag*NDAE1 and *Ag*AE1 exhibit differential expression in distinct compartments of the alimentary canal (Linser et al., 2009) and are the foci of this report.

*Aa*AE1 has been characterized regarding its cDNA sequence, its physiological activity when expressed heterologously in *Xenopus* oocytes and its immunohistochemical localization in adult *Aa* (Piermarini et al., 2010). For the purpose of this comparison between NDAE1 and AE1-type transporters in larval mosquitoes, we have employed an antibody probe previously described which targets the tightly conserved c-terminal amino acid sequence of *Aa*AE1 (Figure 1 and Piermarini et al., 2010). This 22 amino acid sequence is conserved at 15 points between *Aa*AE1 and *Ag*AE1.

NDAE1-like Slc4 transporters have not previously been reported as cloned or characterized from mosquitoes. For this effort, several cDNA-based cloning strategies along with existing EST data were employed to produce several full length cDNA sequences. As is true with a number of Slc4 transporters, we encountered several splice variants of this mRNA. Four of

the full length cDNAs have been submitted to GenBank with accession numbers AY280611 (AgNDAE1-1), FJ981607 (AgNDAE1-2), AB669028 (AgNDAE1-3), and AB669027 (AgNDAE1-4) (Figure 1). Most of the four sequences are identical with a few varied insertions/deletions close to either the N-terminus or the C-terminus. The N-terminus defines a hypothetical intracellular tail of between 378 and 426 amino acids and encompasses the significant insertions/deletions at the N-terminal end of the protein (Figure 1). The C-terminus exhibits a consistent long version and two variable shorter versions in what is another intracellular cytoplasmic tail (Figure 1). The first portion of the C-terminal peptide is consistent between forms (approximately 41 amino acids) with three different patterns thereafter to the terminus. One pattern present in two of the clones is a long tail of 153 to 158 amino acids. The other two clones have distinct short sequences beyond the 41 common to all of either 43 or 18 amino acids. It is possible to speculate that these differences in the cytoplasmic tails reflect regulatory control sequences. Given this variability in the C-terminal domain, we focused on the N-terminus for antibody production and splice variants AgNDAE1-3 and AgNDAE1-4 for functional characterization. Hypothetical models for structure have been published for NDAE1 of *Drosophila* and for Ag (Romero et al.2000, Linser et al. 2009). The internal regions which include the transmembrane regions of both NDAE1 and AE1 from both mosquito species exhibit high homology (Figure 1).

***Anopheles gambiae* NDAE1 (AgNDAE1) is a sodium-driven chloride-bicarbonate exchanger**

To test the function of AgNDAE1, we used the *Xenopus* oocyte expression system and monitored intracellular pH (pH_i) and membrane potential (V_m) using microelectrodes (Figure 2). In these physiological analyses, both variants AgNDAE1-3 and AgNDAE1-4 gave qualitatively similar results. The data shown herein is specifically from AgNDAE1-3. *Drosophila* NDAE1 (dNDAE1) was used as a positive control because it is a likely orthologue of AgNDAE1 and well characterized (Romero et al, 2000). When in CO_2/HCO_3^- buffers, AgNDAE1 (Figure 2A) and dNDAE1 (Figure 2B) mediate HCO_3^- transport in response to removal of Cl^- or Na^+ , i.e., indicative of Na^+ driven Cl^-/HCO_3^- exchange. Fig 2A shows that Cl^- removal ($0Cl^-$) increased pH_i , (Cl^- efflux and HCO_3^- influx; model in Figure 2Ea). By contrast, Na^+ removal elicited a pH_i decrease (Na^+ and HCO_3^- efflux; model in Figure 2Eb). These ion transport modalities are characteristic of the Na^+ -driven Cl^-/HCO_3^- exchange activity reported for *Drosophila* NDAE1 (Figure 2B) (Romero 2000) and mammalian NDCBE (Grichtchenko, 2001) and *C. elegans* atbs-1 (Bellemer et al., 2011). The V_m of the oocytes changed only slightly during these experimental maneuvers, which indicates that AgNDAE transport activity is electroneutral (Figure 2A–C) as reported for dNDAE1 (Romero et al., 2000).

Other halides (e.g. I^- and Br^-) can also be transported in many Cl^- transport systems (Wright and Diamond, 1977). Here we examined I^- transport using anion-sensitive microelectrodes (see Materials and Methods; Bellemer et al., 2011). Oocytes expressing AgNDAE1 showed obvious I^- transport activity (Figure 2D) as did *C. elegans* atbs-1 previously (ibid). By calibrating our anion-sensitive microelectrodes, we determined that the intracellular $[I^-]$ reached over 250 μM within 5 minutes after incubating AgNDAE1-expressing oocytes in high extracellular $[I^-]$. By contrast, control oocytes showed very slight I^- transport (Figure 2D). From this observation, we propose AgNDAE1 transport mechanism as shown (Figure 2Eb). AgNDAE1 transports Na^+ and HCO_3^- out of the cell while transporting halides into the cell (Figure 2Eb).

Antibody probes for AEs

The rabbit antibody probe to AaAE1 (C-terminal peptide) produced striking localization of the transporter to the basal aspect of the stellate cells of the Malpighian tubules (MT) of adult *Aa* (Piermarini et al., 2010). The peptide sequence used in generating this probe (IRKQMERIFSPLRLAL) is identical to the annotated AgAE1 at 15 of 17 amino acids (IRKQLESIFSPLRLAL). Hence there was an initial expectation that the antibody probe would also recognize the orthologous protein in *Ag*. Figure 3 shows that immunohistochemical labeling of adult MTs from both *Aa* and *Ag* exhibit similar specific labeling of the basal aspect of the MT stellate cells. Hence we have used this same antibody to characterize AE1 expression in larvae of both *Aa* and *Ag*.

As shown in Figure 4 (lane 4), rabbit antisera (Q3701 and Q3725) to the 100 amino acid sequence from the N-terminal cytoplasmic domain of AgNDAE1 labeled a polypeptide of molecular mass between 110 kDa and 160 kDa, consistent with an expected molecular mass of ~130 kDa (Figure 4, only results from rabbit Q3701 are shown although both sera produced the same labeling pattern). For this analysis, the total membrane fraction from *Ag* larval MTs was prepared as described (Umesh et al., 2003) and used as test material. A very high (>260 kDa) molecular mass blur of staining was evident in immunoblots and we interpret that to be un-dissociated macro aggregates of the NDAE1 protein or glycosylation variants strikingly similar to details reported for AaAE1 from adult MTs (Piermarini et al., 2010). Faint laddering lower molecular mass bands are also observed which most likely represent degradation products. Preimmune sera from both rabbits produced no staining (Figure 4 lane 3). To further test the specificity of the antisera, *Xenopus* oocytes were injected with 25 ng of cRNA for the two cDNAs (AgNDAE1-3 & 4) and allowed 3 days for protein expression. Figure 5 shows a summary of immunostaining results. Both antisera labeled the NDAE1-mRNA injected oocytes whereas water injected oocytes showed no signal as was also evident in mRNA injected oocytes probed with preimmune serum from both rabbits. Figure 5 shows the results specifically for AgNDAE1-3 but both constructs (AgNDAE1-3 & 4) gave the same pattern of immunolabeling in the injected oocytes.

When the AgNDAE1 antisera were tested in similar analyses against *Aa* Malpighian tubule proteins via immunoblotting, no specific bands were detected. Similarly, no specific immunostaining of *Aa* tissues was detected in immunohistochemical analyses.

The distribution of the NDAE1 and AE1 proteins in larval tissues was examined using both whole mount preparations and histological sections of 4th instar larvae. Since both the AaAE1 and the AgNDAE1 antibodies were produced in rabbits, multiple labeling paradigms with a variety of counter markers were used as a strategy to establish the relationship between the distributions of these two anion transport proteins. Figure 6 shows that as in adult *Aa*, the AaAE1 antibody labels the stellate cells of the MTs in larvae of both *Aa* and *Ag*. The labeling is specific to the basal membranes of the stellate cells and does not seem to have a distributional variability along the proximal to distal extremes of the individual MTs (Figure 6). The stark intensity of labeling of the stellate cells in contrast to a nearly blank background in other tissues of the posterior alimentary canal is very striking. The anterior region of the alimentary canal however exhibits very pronounced labeling of many (but not all) of the cells of the lobes of the gastric caecae (GC, Figure 7). In Figure 7 the GC regions of the anterior alimentary canal of both species are compared and the analysis of antigen distribution facilitated by comparison to the distribution of Na⁺/K⁺-ATPase (subunit alpha, Patrick et al., 2006, Smith et al., 2007). In both *Aa* and *Ag* larval GC, labeling for AE1 is found associated with many of the GC cells but in a patchwork pattern indicating a differential pattern of expression among the individual cells of the GC. In *Aa*, the posterior-most grouping of cells on each of the 8 GC lobes have been termed “CAP” cells in their distinction from other GC cells (Seron et al., 2004). In the current context, the CAP cells of

Aa exhibit the highest levels of AE1 protein. (Fig 7, B and C, E and F). In *Ag* the GC labels in a similar patchy pattern with varying intensity among cells. Cells located toward the posterior extreme of each GC lobe which are possibly analogous to the *Aa* CAP cells label strongly for AE1. In both *Aa* and *Ag* examination of cross-sectional views show that the localization of AE1 is to the basal membrane as it co-localizes with Na^+/K^+ -ATPase (an established marker of basal membranes in GC, Patrick et al., 2006; Figure 7, D–F and J–L). It should be noted that the Na^+/K^+ -ATPase labeling is also not uniform among GC cells (Patrick et al., 2006). Indeed, the CAP cells of *Aa* are remarkable in their lack of this marker (ibid) whereas other AE1-labeled GC cells do possess the ATPase as well (Fig 7). AE1 is also detected on the basal membranes of anterior midgut and posterior midgut cells but to levels much lower than that seen in the GC (data not shown).

The localization of AgNDAE1 provides a very stark contrast to that of AE1. Figure 8 shows histological sections of *Ag* larval hind gut regions of the alimentary canal. One cell type stands out as the primary site for expression of this protein in the posterior gut and that is the principal cell of the MT (Fig 8). Whole mounts (not shown) and histological sections show very robust labeling of the principal cells on the basal plasma membrane. Contrasted with V-ATPase (apical microvilli marker for principal cells and the apical aspect of other hindgut cells [Zhuang et al., 1999; Patrick et al., 2006]) the specificity of AgNDAE1 accumulation at the basal membrane of the MTs is evident (Fig 8). Additionally, these analyses make it clear that the stellate cells, the sites of AE1 expression, show no detectable NDAE1 antigen (Fig 8, A). As with AE1, little NDAE1 immunoreactivity is detected in other areas of the alimentary canal except for the GC. Figure 9 shows low and high magnification views of larval *Ag* anterior gut region. Prominent staining for AgNDAE1 is seen only on the apical microvilli of certain GC cells. When NDAE1 staining is contrasted with that of CA9 and Na^+/K^+ -ATPase, the AgNDAE1 appears to be most strongly associated with GC cells that are strongly positive for CA9 and for Na^+/K^+ -ATPase and absent from those lacking these counter markers (Fig. 9E–H).

It should be noted that the expression of AE1 and NDAE1 proteins is not solely limited to the cells and tissues described here although the Malpighian tubules and the gastric caecae of larvae are the most intense localizations. AE1 immunoreactivity is seen at low levels on the basal membranes of other cells of the larvae, such as the basal membranes of the posterior midgut cells. NDAE1 protein is also expressed at high levels in very specific regions of the nervous system.

Discussion

The present study reports the first characterization of a mosquito NDAE1-type AE (AgNDAE1). The amino acid sequence of AgNDAE1 exhibits very high similarities to dNDAE1 cloned by Romero et al., (2000). To wit, using pH_i measurements of *Xenopus* oocytes expressing AgNDAE1, we found a pattern of activity that is qualitatively very similar to the characteristics of the archetypical dNDAE1 (Romero et al., 2000; Sciortino et al., 2001). That is, AgNDAE1 appears to be a true molecular and functional orthologue of dNDAE1 and thus likely cotransports Na^+ and 2HCO_3^- in electroneutral exchange with Cl^- (Sciortino, 2001). Thus, while V_m is not a driving force for NDAE1 transport, intracellular and extracellular ion activities Na^+ , Cl^- , and HCO_3^- are. That is, net changes in the concentration gradients of these ions, can change the direction of NDAE1-mediated transport (e.g. NaHCO_3 into the cell vs. NaHCO_3 out of the cell). This biophysical and physiological flexibility means that the other net ion movements in the GC or MT can respond to changes in the local environment (i.e. homeostasis). Unknown however, is whether AgNDAE1 is an obligate HCO_3^- transporter or whether pseudo-halides (NO_3^- or OH^-) could result in Na^+ exchange for Cl^- . This later possibility raises the question that if

AgNDAE1 can transport CO_3^{2-} , are other divalent anions such as sulfate (SO_4^{2-}) also transported? Given that chondroitin sulfate synthesis is required for *Plasmodium* invasion of the Ag gut (Dinglasan et al., 2007), Ag gut SO_4^{2-} transporters become important anti-mosquito and anti-malaria drug targets

The cell biology of the larval alimentary canal is undeniably complex. Each of the major structural domains exhibits cellular specializations and diversity even though throughout the length of the organ system the tissue is essentially a single-celled epithelium (Clements, 1992). The immunohistochemical analyses that we performed using specific probes for the AE1 and NDAE1 Slc4 transporters identified two regions with the highest levels of expression of the proteins: the Malpighian Tubules (MT) and the Gastric Caecae (GC). The five MTs of the mosquito are considered to be major contributors to salt and water balance of the extracellular fluid (hemolymph) and the excretion of wastes from the hemolymph (Dow, 1984; Beyenbach, 2003; Romero et al., 2004; Piermarini et al., 2010; Beyenbach and Piermarini, 2009, 2011). The facility with which adult mosquito MTs can be studied in isolation from the organism has produced numerous physiological analyses (Ramsey, 1950 and *ibid*). In contrast, larval MTs are less well studied and the fact that they are the only portions of the larval alimentary canal to be maintained into the adult stage suggests that their functionality is similar between life stages. Consistent with this assumption is the localization of AE1 immunoreactivity to the basal membrane of the stellate cells in Malpighian tubules of both adults and larvae in both *Aa* and *Ag*. No AE1 immunoreactivity is detected in the other major cell type in the mosquito MT, the principal cell which makes up the vast majority of the actual tissue mass (Beyenbach and Piermarini, 2009). In striking contrast to the stellate cell-specific expression of AE1, AgNDAE1 appears to be very specific to the basal aspect of the principal cells, without detectable expression in stellate cells (at least in MTs of *Ag* larvae). Our antisera to AgNDAE1 did not detect NDAE1 immunoreactivity in comparable tissues of larval *Aa*, which reflects either (1) a more limited expression of the protein in *Aa* vs *Ag*, or (2) a poor cross-reactivity of the Q3701 and Q3725 antisera with AaNDAE1. The latter may be likely given that the amino acid sequences of the AgNDAE1 and the putative AaNDAE1 antigenic sites differ in 19 of the 100 amino acids and AaNDAE1 is missing an additional 3 amino acids in this region.

The physiological activities and homeostatic contributions of insect MTs has been the subject of numerous investigations. Physiological models have been proposed by several laboratories. Indeed, a new model incorporating several regulatory proteins discussed herein is presented in Xiang et al., (this issue). Finding robust expression of NDAE1 and AE1 in larval AgMTs indicates that the MTs likely play a significant role in the regulation of hemolymph HCO_3^- and pH in larval mosquitoes. Moreover, there is likely a specialization in the handling of HCO_3^- by the principal cells and the stellate cells. For example, the presence of a basal acid-loader such as AE1 in stellate cells suggests that these cells may contribute to the re-absorption of HCO_3^- by larval MTs, whereas the presence of a basal acid-extruder such as NDAE1 in the principal cells suggests that these cells may contribute to the secretion of HCO_3^- by larval MTs. These are, of course, hypotheses that will require testing in following studies.

A second domain of robust expression of both AgAE1 and AgNDAE1 was identified in the alimentary canal in the cells of the GC. The classical literature characterizes the cells of the GC as participating in ion regulatory events as well as food digestion and nutrient absorption (Volkman and Peters, 1989a&b; Clements, 1992). Microarray analyses confirm these hypotheses and specifically identify genes associated with these functions (Neira-Oviedo et al., 2008). It has also been evident for many years that the GC epithelium is composed of cell types that seem cytologically to have differing specializations (Volkman and Peters, 1989a). Our studies add to this picture of complexity. The AaAE1 antibody probe showed

the presence of strongly positive cells in a patchwork around any one of the eight lobes of the GC region of the alimentary canal. In all cases and in both mosquito species, the AE1 reactivity was basal membrane-specific but with the intensity of labeling different even between neighboring cells. In *Aa*, the posterior cells of each caecal lobe have been named “CAP” cells because of distinctions in structure and marker expression (Seron et al., 2004). In the current analysis it was evident that these CAP cells express much higher levels of AE1 than do other cells of the GC. In *Ag*, AE1 localization similarly showed cell heterogeneity but the striking position and grouping of CAP cells is less evident (by these and other analyses, unpublished results). Nonetheless, as CAP cells in *Aa* exhibit little or no expression of the cytoplasmic carbonic anhydrase CA9, some of the more posterior GC cells of *Ag* show little or no CA9 but very high levels of AE1. The other GC cells that do exhibit very high levels of CA9 do also show appreciable basal AE1 but to a lower level than in the CAP/CA9-negative cells. *Aa* CAP cells and their presumed counterpart in *Ag* share the distinction of expressing little or no basal Na^+/K^+ -ATPase but high levels of V-ATPase in an apparently intracellular domain. In contrast, AgNDAE1 exhibits apical expression on the GC cells that are not similar to the *Aa* CAP cells. These cells exhibit high cytoplasmic CA9, moderate to high basal AE1, moderate to high basal Na^+/K^+ -ATPase and high apical V-ATPase. Although we did not attempt direct labeling of the AE1 and NDAE1 rabbit antibody probes which would have allowed for simultaneous localization of the two anion exchangers, the cross-correlations between other antigens indicate that in fact NDAE1 and AE1 can occur in the same cell type but on opposite sides (apical versus basal) of a given cell. Direct proof of this contention will be necessary to allow construction of a working model at the level of the individual cell.

Very little is known about the physiological roles of the GC cells of the larval mosquito alimentary canal. Transcriptomic analyses provides a glimpse into highly enriched mRNAs and hence a superficial look at cell and tissue specialization (Neira-Oviedo et al., 2008; Linser, unpublished results). The analyses presented herein show that GC cells are heterogeneous and that different cell types express ion regulatory functionalities in distinct patterns. Robust expression of the Slc4 transport proteins indicates roles in anion regulation that is somehow balanced with the vectorial positioning of such cation transporters as V-ATPase and Na^+/K^+ -ATPase. Modeling of the roles of the GC in gut function and animal homeostasis will require detailed mapping of such activities and their interrelationships at the level of individual cells. The very high level of expression of ion regulatory proteins in the GC implies a central role in the physiological balance of the alimentary canal and indeed pH regulation in general. The presence of NDAE1 on the apical brush border membranes of many GC cells provides a possible mechanism for diverting HCO_3^- into the lumen of the gut. The musculature of the GC lobes contracts and produces a pulsatile movement of fluids from the GC lumen into the stomach (Jones, 1960). Perhaps this may contribute to the high levels of $\text{HCO}_3^-/\text{CO}_3^{2-}$ in the gut lumen which is thought to buffer the pH extremes measured therein (Boudko et al., 2001; Linser et al., 2009). As in the MTs, a clear understanding of the interactions and dynamic properties of the ion regulatory proteins of the GC will be necessary to produce a comprehensive understanding of tissue function. The significance of multiple AE and NDAE1 transcripts is unknown (Figure 1). The present results indicate that the AgNDAE1-3 and the AgNDAE1-4 have similar function. The antibodies used in this study do not discriminate these proteins, so tissue specific isoform expression as well as membrane localization could account for some differences in GC and MT physiology, which remains to be more fully elucidated.

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HIGHLIGHTS

- Slc4-like transporters show specific patterns of expression in larval mosquito gut
- NDAE1 orthologue in *An. gambiae* larvae is basal in Malpighian tubules and apical in gastric caecae
- AE1 orthologue in *An. gambiae* larvae is basal in Malpighian tubules and in gastric caecae
- Malpighian tubule stellate and principal cells express distinct Slc4-like anion transporters
- *An. gambiae* NDAE1 is physiologically similar to *Drosophila* NDAE1, a Na-driven Cl/HCO₃ exchanger

AgtDAE1-1 : :
 AgtDAE1-2 : :
 AgtDAE1-3 : :
 AgtDAE1-4 : :
 AnMDA1-A : :
 AnMDA1-B : :
 AaA1 : :
 AaA1-A : :
 AaA1-B : :
 AgA2 : :
 AgtDAE1-1 : : 39
 AgtDAE1-2 : : 39
 AgtDAE1-3 : : 39
 AgtDAE1-4 : : 39
 AnMDA1-A : : 115
 AnMDA1-B : : 93
 AaA1 : : 190
 AaA1-A : : 190
 AaA1-B : : 190
 AgA2 : : 8
 AgtDAE1-1 : : 94
 AgtDAE1-2 : : 94
 AgtDAE1-3 : : 94
 AgtDAE1-4 : : 115
 AnMDA1-A : : 93
 AnMDA1-B : : 93
 AaA1 : : 190
 AaA1-A : : 190
 AaA1-B : : 190
 AgA2 : : 38
 AgtDAE1-1 : : 179
 AgtDAE1-2 : : 179
 AgtDAE1-3 : : 179
 AgtDAE1-4 : : 200
 AnMDA1-A : : 175
 AnMDA1-B : : 175
 AaA1 : : 190
 AaA1-A : : 190
 AaA1-B : : 190
 AgA2 : : 129
 AgtDAE1-1 : : 255
 AgtDAE1-2 : : 255
 AgtDAE1-3 : : 255
 AgtDAE1-4 : : 255
 AnMDA1-A : : 249
 AnMDA1-B : : 249
 AaA1 : : 469
 AaA1-A : : 469
 AaA1-B : : 469
 AgA2 : : 292
 AgtDAE1-1 : : 329
 AgtDAE1-2 : : 329
 AgtDAE1-3 : : 329
 AgtDAE1-4 : : 336
 AnMDA1-A : : 321
 AnMDA1-B : : 321
 AaA1 : : 487
 AaA1-A : : 487
 AaA1-B : : 487
 AgA2 : : 320
 AgtDAE1-1 : : 428
 AgtDAE1-2 : : 428
 AgtDAE1-3 : : 428
 AgtDAE1-4 : : 428
 AnMDA1-A : : 420
 AnMDA1-B : : 420
 AaA1 : : 587
 AaA1-A : : 587
 AaA1-B : : 587
 AgA2 : : 326
 AgtDAE1-1 : : 503
 AgtDAE1-2 : : 503
 AgtDAE1-3 : : 503
 AgtDAE1-4 : : 510
 AnMDA1-A : : 495
 AnMDA1-B : : 495
 AaA1 : : 578
 AaA1-A : : 578
 AaA1-B : : 578
 AgA2 : : 426

Figure 1. Mosquito Slc4 protein line-ups

ClustalW-2 was utilized to produce an alignment of the mosquito SLC4 family members from *Anopheles gambiae* (Ag) and from *Aedes aegypti* (Aa): AgNDAE1-1 (splice variant-1, AY280611); AgNDAE1-2 (splice variant-2, FJ981607); AgNDAE1-3 (splice variant-3, AB669027); AgNDAE1-4 (splice variant-4, AB669028); AaNDAE1-A (splice variant-A, EAT37256); AaNDAE1-B (splice variant-B, EAT37255); AaAE1 (EU700988); AgAE1-A (splice variant-A, EAL40007, not fully annotated); AgAE1-B (splice variant-B, EDO63795, not fully annotated); AgAE2 (EU068741). The alignment was visualized using GeneDoc software with shading set in “conservation mode” so that black, dark grey, light grey and white backgrounds indicate from highest to lowest levels of conservation between sequences. Putative transmembrane regions were predicted using SOSUI (<http://bp.nuap.nagoya-u.ac.jp/sosui>) and highlighted with a solid black line over-score. The 100 amino acid peptide sequence used to generate the AgNDAE1 antibody probes is depicted in the sequence line for AgNDAE1-2 with five discontinuous open box over-scores. The antigenic peptide used to produce the antibody probe for AaAE1 is indicated with an open box on the AaAE1 sequence line near the carboxy terminus. The sequences for AgNDAE1 (1–4) and AgAE2 were all generated as full-length PCR products from a cDNA library. The AaAE1 sequence was also from a full length cDNA as previously described (Piermarini et al, 2010). The other sequences presented were taken from genome data bases as assembled and annotated from combined sources.

Figure 2. AgNDAE1 is a sodium-driven chloride-bicarbonate exchanger

(A–C) Electrophysiology data using pH microelectrodes in *Xenopus* oocytes. In the presence of 5% CO₂/33 mM HCO₃⁻ (pH 7.5), both AgNDAE1 (A) and *Drosophila* NDAE1 (B) responded to Cl⁻ replacement (0Cl⁻) by increasing pH_i (HCO₃⁻ influx to oocyte), while water-injected oocytes (C) showed no response. By contract, Na⁺ removal (0Na⁺) resulted in dramatic pH_i decreases (HCO₃⁻ efflux from oocyte) in oocytes expressing *Anopheles gambiae* (A) and *Drosophila* NDAE1 (B), while water-injected oocytes (C) showed no response. Rates of alkalization during Cl⁻ and Na⁺ removal ($\# \times 10^{-5}$ pH units/s) are indicated above the respective segments of the pH_i traces. (D) Electrophysiology data using Cl⁻ (halide) microelectrodes in *Xenopus* oocytes. In oocytes expressing AgNDAE1 and water-injected controls, replacement of extracellular Cl⁻ with gluconate produced no change in [Cl⁻]. When extracellular Cl⁻ was replaced completely by I⁻, the electrode sensed I⁻ influx in oocytes expressing AgNDAE1, but not in water-injected controls. (E) Models illustrating the suggested transport activity of *Anopheles gambiae* and *Drosophila* NDAE1 in response to removal of extracellular Cl⁻ (a) and in response to removal of extracellular Na⁺ or addition of extracellular I⁻ (b). The transport activity illustrated in panel (Ea) is also the predicted activity of AgNDAE1 under normal conditions. The stoichiometry shown, with two HCO₃⁻ ions transported, is consistent with the observed electroneutrality of the transport.

Figure 3. AE1 in adult *Aa* and *Ag*

The antibody probe for AaAE1 (Piermarini et al., 2010) was tested against adult *Aa* and *Ag* to ensure cross-reactivity. Panel A shows hindgut region of an adult female *Aa* whole mount immunostained with the AE1 probe. Note the expected staining of stellate cells (exemplified at arrows) throughout the Malpighian tubules. A similar whole mount of an adult female *Ag* is shown in B with stellate cells of the Malpighian tubules exemplified by the arrows. “R” indicates the location of the rectum and “IL” the ileum. Nuclei were stained with DAPI and are evident throughout both preparations as large and small grey circular profiles in this black and white image.

Figure 4. (Immunoblot of AgNDAE1)

SDS-PAGE of total membrane proteins from 4th instar larval Ag MTs. Lanes 1 and 2 show Fast Green Staining of either molecular mass markers (masses indicated at left, 1) or membrane proteins (2). Lane 3 shows a blot that was probed with preimmune rabbit serum from the Q3701 rabbit and lane 4 shows a parallel lane probed with Q3701 antiserum diluted 1:1000 and detected with alkaline phosphatase-conjugated goat anti rabbit developed with AP chemistry. Arrow indicates primary band consistent with expectations.

Figure 5. (Oocyte staining)

Frozen sections of *Xenopus* oocytes that had been injected 3 days earlier either with 25 ng cRNA encoding AgNDAE1 (A,C,D) or with water (B). Note labeling of oocyte surface with the Q3701 antiserum (A [low magnification], C, D [hi magnification]) and absence of staining in the water injected oocyte (B). D shows a merge of immunostaining fluorescence with differential interference contrast image.

Figure 6. (AE1 in posterior alimentary canal of *Aa* and *Ag*)

Whole mount preparations of isolated and formaldehyde fixed alimentary canal tissues from *Aa* (A and B) and *Ag* (C and D) larvae immunostained with the AaAE1 antibody (Piermarini et al., 2010). Shown is the hindgut region including the Malpighian tubules, rectum (R), ileum (IL), and a portion of the posterior midgut (PMG). White highlights (A–D) indicates localization of the AaAE1 immunoreactivity in these laser scanning confocal images and grey circular profiles (only in B & D which is an overlay of both immunofluorescence and DAPI staining) indicating cellular nuclei. Note the stellate pattern of staining for AaAE1 along the length of each Malpighian tubule.

Figure 7. (AE1 staining of anterior alimentary canal [GC region])

Whole mount preparations of isolated and formaldehyde fixed alimentary canal tissues from *Aa* (A–F) and *Ag* (G–L) larvae immunostained with the AaAE1 antibody (Piermarini et al., 2010). Panels A and G show DIC images of the respective tissues shown in this figure. Panels B and H show maximum projections of a z stack of scans for the total depth of the tissues with C and I being merged images of three markers shown here. Red staining represents AaAE1 immunostaining and blue represents Na^+/K^+ -ATPase while yellow/gold represents DAPI (nuclei). Panels D–F and J–L show single z planes of focus from each stack. Long arrows in B, D, E, and F indicate position of *Aa* posterior GC CAP cells. Short arrows in D, E, F, J, K and L indicate examples of locations of colocalization of AaAE1 and Na^+/K^+ -ATPase immunoreactivity.

Figure 8. (AgNDAE1 immunostaining of posterior alimentary canal)

Histological sections of *Ag* larval hindgut tissues immunostained for AgNDAE1 (red), V-ATPase (green) and CA9 (blue). In A, a single MT is shown in longitudinal section with prominent AgNDAE1 labeling of the basal side of the principal cells and V-ATPase labeling of the cytoplasmic and apical membrane compartments of the principal cells. Note the arrow which indicates a stellate cell lacking staining for with marker. The rectum (R) which is composed of DAR (CA9-positive) and non-DAR cells (V-ATPase-positive, Smith et al., 2007) is evident to the right in A. Panel B (confocal maximum projection) and C (fluorescence and DIC merged single z plane) show a higher magnification view in cross section of a MT with evident AgNDAE1 labeling at the basal surface and V-ATPase at the apical brush border membrane of the principal cells.

Figure 9. (AgNDAE1 immunostaining of GC)

Histological sections of *Ag* larval anterior alimentary canal region immunostained for AgNDAE1 (green), CA9 (blue) and Na⁺/K⁺-ATPase (red). Panels A – D show a low magnification view of a maximum projection z stack. The head of the larva is at the top of the image. AgNDAE1 staining is evident in A and the merged image in D associated with the apical brush border membranes of certain cells of the GC. At higher magnification (E–H), the brush border microvilli of certain GC cells are heavily labeled for AgNDAE1 (long arrows). The short arrow indicates cells of the posterior extreme of the GC lobe that do not label for any of the three markers examined in this analysis. “Stem” refers to a group of cells that are part of the connection between the anterior midgut and the GC lobe. Panel H is a merge of the fluorescence channels and DIC of a single plane from the z stack.

Table 1

Comparison of mosquito Slc4 nomenclature.

	Linser et al 2009	Present study
Sodium-coupled	AE1	NDAE1
Sodium-independent	AE2	AE1
Sodium-independent	AE3	AE2