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Review

NHE_{VNAT}: an H^+ V-ATPase electrically coupled to a Na^+:nutrient amino acid transporter (NAT) forms an Na^+/H^+ exchanger (NHE)

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Summary

Glycolysis, the citric acid cycle and other metabolic pathways of living organisms generate potentially toxic acids within all cells. One ubiquitous mechanism for ridding cells of the acids is to expel H^+ in exchange for extracellular Na^+, mediated by electroneutral transporters called Na^+/H^+ exchangers (NHEs) that are driven by Na^+ concentration gradients. The exchange must be important because the human genome contains 10 NHEs along with two Na^+/H^+ antiporters (NHAs). By contrast, the genomes of two principal disease vector mosquitoes, *Anopheles gambiae* and *Aedes aegypti*, contain only three NHEs along with the two NHAs. This shortfall may be explained by the presence of seven nutrient amino acid transporters (NATs) in the mosquito genomes. NATs transport Na^+ stoichiometrically linked to an amino acid into the cells by a process called symport or co-transport. Three of the mosquito NATs and two caterpillar NATs have previously been investigated after heterologous expression in *Xenopus laevis* oocytes and were found to be voltage driven (electrophoretic). Moreover, the NATs are present in the same membrane as the H^+ V-ATPase, which generates membrane potentials as high as 120 mV. We review evidence that the H^+ V-ATPase moves H^+ out of the cells and the resulting membrane potential (V_m) drives Na^+ linked to an amino acid into the cells *via* a NAT. The H^+ efflux by the V-ATPase and Na^+ influx by the NAT comprise the same ion exchange as that mediated by an NHE; so the V and NAT working together constitute an NHE that we call NHE_{VNAT}. As the H^+ V-ATPase is widely distributed in mosquito epithelial cells and there are seven NATs in the mosquito genomes, there are potentially seven NHE_{VNAT}s that could replace the missing NHEs. We review published evidence in support of this hypothesis and speculate about broader functions of NHE_{VNAT}s.

Key words: electrogenic, electrophoretic, KAAT1, CAATCH1, *Ae*AAT1i, *Ag*NAT8.

Introduction

How cells get rid of metabolically produced acids has been an intriguing question for more than half a century. Murer and colleagues first reported a Na^+/H^+ antiport in brush-border membrane vesicles (BBMVs) from rat small intestine and kidney (Murer et al., 1976). Pouysségur and associates cloned the first Na^+/H^+ exchanger (NHE) from a human cDNA collection (Sardet et al., 1989). Presently, the ability to eject cellular H^+ by exchange with external Na^+ mediated by NHEs is thought to occur in all eukaryotic cells. The energy for the exchange is furnished by ATP hydrolysis and is mediated by the Na^+/K^+ P-ATPase, which typically is located on the cell's basolateral membrane where it expels Na^+, keeping the cell Na^+ concentration low. NHE is also located on the cell's basolateral membrane in mammals where it uses the large Na^+ gradient to import Na^+ and export H^+. The stoichiometry of the exchange is: 1 Na^+ in, 1 H^+ out, so NHE is electroneutral and the only force driving the cation exchange is the Na^+ concentration gradient (Orlowski and Grinstein, 2004).

Gill and associates (Pullikuth et al., 2006) cloned the Na^+/H^+ exchanger 2 from *Aedes aegypti* (*Ae*NHE2) (*Ae*NHE3 in their terminology) and characterized it heterologously in yeast cells where it complements mutants deficient in certain NHEs; they also expressed it in NHE-deficient fibroblast cells where it enabled the recovery of intracellular pH following an acid load. In mosquitoes, *Ae*NHE2 is located on the basal membranes of Malpighian tubules and midgut cells where it appears to play a role in pH regulation. More recently, Kang'ethe, Gill and associates (Kang'ethe et al., 2007) cloned the Na^+/H^+ exchanger 1 from *Ae. aegypti* (*Ae*NHE1) (*Ae*NHE8 in their terminology). When heterologously expressed in NHE-deficient yeast cells, it restored the ability to grow in high NaCl medium. In proteoliposomes carrying yeast membranes, it mediated the exchange of Na^+ or K^+ for H^+. In mosquito adults, it was localized to the apical plasma membranes in Malpighian tubules, gastric caeca (GC) and the rectum. Gill's group proposed that in Malpighian tubules, *Ae*NHE1 couples the inward H^+ gradient created by the H^+ V-ATPase to extrude excess Na^+ and K^+ 'while maintaining steady intracellular pH in the principal cells'.

An entirely different mechanism for Na^+/H^+ exchange between cells and lumen involves the interaction between two membrane proteins: (1) an H^+ V-ATPase that translocates H^+ across the lipid bilayer toward the lumen and generates a transmembrane voltage (lumen positive); and (2) a voltage-driven, (Na^+ or K^+)-coupled nutrient amino acid transporter (NAT) that moves Na^+ linked to an amino acid into the cells. A much-studied example is the symport

of essential amino acids from the midgut lumen into the epithelial cells of caterpillars. Nedergård first demonstrated active amino acid uptake by the isolated caterpillar midgut and showed that it is voltage dependent (Nedergård, 1972a). Hanozet, Giordana and Sacchi (Hanozet et al., 1980) demonstrated K^+:amino acid symport in BBMV from wild silkworm midgut and initiated a series of studies that culminated in the identification of six K^+-coupled amino acid uptake systems (Giordana et al., 1989). Meanwhile, Ramsay, Harvey, Gupta and Berridge, and Maddrell and others had identified and characterized a so-called electrogenic K^+-pump, and Cioffi, Wolfersberger and Harvey had isolated the goblet cell apical membrane (GCAM) in which the K^+-pump resides and showed that it is an ATPase [Harvey et al. (Harvey et al., 1983) and references therein]. Wieczorek, Klein and associates solubilized the GCAM ATPase and showed that it is an H^+ V-ATPase (Wieczorek et al., 1989). It is now clear that all H^+ V-ATPases are electrogenic membrane energizers that hyperpolarize the membranes in which they reside (reviewed by Nelson and Harvey, 1999).

Recall that the V-ATPase moves H^+ from cell to lumen and the NAT moves Na^+ from lumen to cell, like classical NHEs. Research has focused on the uptake of amino acids rather than Na^+ and no one recognized this new, electrically coupled method for Na^+/H^+ exchange until Harvey, Okech and associates brought attention to it (Okech et al., 2008a). They noted the location of the H^+ V-ATPase and a Na^+:amino acid transporter (*Ag*NAT8) together on the apical plasma membrane of the epithelial cells in GC and posterior midgut (PMG) of *Anopheles gambiae* larvae. They deduced that the H^+ V-ATPase, *Ag*NAT8 pair acts like an NHE and suggested the term NHE_{VNAT}. Critics objected that the deduction is too obvious and that the term is unnecessary. We counter that a phenomenon that had remained unrecognized for a quarter of a century deserves a name. To quote the closing line in Peter Mitchell's 1978 Nobel lecture 'The obscure we see eventually, the completely apparent takes longer'. No one doubts that H^+ V-ATPases are voltage-generating (electrogenic) H^+ exporters. We will review the evidence that many amino acid transporters are voltage-driven (electrophoretic) Na^+ or K^+ importers.

Review and perspective

General principles of electrophoretic transporters

Many secondary solute transporters are electrophoretic

Electrical coupling between membrane proteins is a topic that is seldom discussed. For one thing, the terminology is confusing. Transporters that generate electricity under laboratory conditions are usually electrically driven in living organisms, so they are electrophoretic not electrogenic. As the literature on solute transporters grows, the distinction between proteins that generate membrane potentials (V_m) and those that use the potentials to drive solute transport becomes more important. For example, the H^+ V-ATPase of eukaryotes is purely electrogenic – its sole action is to translocate H^+ across a membrane's dielectric lipid bilayer and charge its capacitance, resulting in a V_m. Whether the output side is acidified, remains pH neutral or is alkalinized depends upon other components in the membrane, thus: V_m could drive Cl^- through a Cl^- channel and acidify the output compartment; V_m could not affect an electroneutral NHE or the pH but V_m could drive the co-transport of Na^+ linked to an amino acid away from the lumen *via* an electrophoretic symporter – the replacement of Na^+ by H^+ from the ATPase would tend to acidify the lumen (Harvey, 1992). For example *Ag*NAT8 is an insect symporter that uses the V_m generated by an H^+ V-ATPase to drive Na^+ stoichiometrically linked to phenylalanine or tyrosine into cells (Meleshkevitch et al., 2006). The H^+ that had been translocated across the lipid bilayer by the V-ATPase would replace the Na^+ in the lumen and the pH would change in the acid direction. Insects often use K^+ rather than Na^+ during secondary transport but we will retain terms like NHE, Na^+/H^+ antiporters (NHA), Na^+:amino acid symport, understanding that K^+ is often substituted.

Voltages *vs* concentration gradients as membrane energizers

The voltage gradients across biomembranes are enormous. For example, in the caterpillar midgut the phosphorylation potential is ~240 mV (Mandel et al., 1975) and voltages nearly equal to this amount were reported by Dow and Peacock (Dow and Peacock, 1989). This value is 3–4 times the V_m commonly reported across the majority of animal plasma membranes and nearly double the voltage observed across the mitochondrial inner membrane. 240 mV is equivalent to a 10,000-fold concentration gradient across a membrane for a monovalent ion such as Na^+. In mosquito midgut, the potential difference may be ~120 mV, which is equivalent to a 100-fold concentration gradient for a monovalent ion. Thus, the voltage gradients generated by H^+ V-ATPase membrane energization are enormous and to ignore them is to miss a large part of membrane biology.

Essential amino acids require membrane proteins to enter cells

Between 10 and 12 amino acids cannot be synthesized within most metazoan cells and must be taken up from the diet. As amino acids are polar and charged, they cannot simply diffuse across the lipid bilayer of plasma membranes and several classes of membrane transport proteins that mediate their movements have evolved. Membrane proteins that facilitate the diffusion of amino acids down their own electrochemical gradients are called uniporters. Those that mediate ion uptake stoichiometrically linked to solute uptake are called co-transporters by vertebrate physiologists and symporters by those who work on insects or prokaryotes. A symporter can be Na^+ or K^+ concentration gradient driven, or voltage driven. In marine animals and their descendents, Na^+ is the common coupling ion but in caterpillars and fresh water dwellers, such as mosquitoes, K^+ may be the preferred ion.

Basal and lateral *vs* basolateral membranes in insect epithelia

Vertebrate epithelial cells are characteristically joined by tight junctions that define two compartments – a luminal compartment outside the apical membrane and an extracellular fluid compartment outside the basolateral membrane. Insect epithelial cells have a series of septate junctions (Oschman and Berridge, 1971) that hold the cells together all along their lateral surfaces and define a lateral membrane. Separate lateral membranes were isolated from basal membranes (Cioffi and Wolfersberger, 1983) and exhibited distinct patterns on SDS gels (Wieczorek et al., 1990). Accordingly, we will use the terms apical, lateral and basal membranes.

Early studies on caterpillar amino acid transport

Electrophoretic amino acid uptake in caterpillars

From the outset it was clear that amino acid uptake in the isolated midgut of caterpillars is voltage dependent (Nedergaard, 1972a; Nedergaard, 1972b). By the 1990s several major amino acid transport systems had been characterized in BBMV from lepidopteran midgut, including, but not limited to, *Philosamia cynthia* (Hanozet et al., 1980; Giordana et al., 1982; Giordana and Parenti, 1994), *M. sexta* (Hennigan et al., 1993b; Hennigan et al., 1993a; Reuveni et al., 1993; Bader et al., 1995; Liu and Harvey,

1996a; Liu and Harvey, 1996b; Liu et al., 2003), *Bombyx mori* (Parenti et al., 2000; Leonardi et al., 2001a; Leonardi et al., 2001b) and *Pieris brassica*e (Wolfersberger et al., 1987) (reviewed by Giordana and Parenti, 1994; Castagna et al., 1997; Wolfersberger, 2000; Boudko et al., 2005b). Passive facilitative diffusion systems are widespread but the predominant mechanisms are K^+ or Na^+ dependent, neutral and cationic amino acid transporters, which are driven mainly by the V_m.

In their studies on the intestinal BBMV from lepidopteran larvae, the Italian workers identified six K^+ coupled amino acid transport systems in the apical membrane of epithelial cells in the caterpillar midgut. Giordana and Parenti (Giordana and Parenti, 1994) wrote... 'These systems are 1) a neutral amino acid transporter with a broad spectrum of interactions with most neutral amino acids, which is highly concentrative, strongly K^+- and electrical potential-dependent, poorly stereospecific, and recognizes histidine, but not proline, glycine, or alpha-(methylamino) isobutyric acid (MeAIB); 2) a specific system for L-proline; 3) a specific system for glycine with a higher affinity for Na^+ than for K^+; 4) a specific system for L-lysine, which is dependent on membrane potential, is highly sensitive to external K^+, and does not interact with L-arginine or neutral amino acids; 5) a specific K^+-dependent process for glutamic acid, which does not recognize aspartic acid; and last, 6) an apparently unique K^+-driven mechanism for D-alanine, which is potential-dependent and strongly stereo specific.'

Energization of amino acid transport in mosquito larvae

In mosquito larvae, 10 L-amino acids are essential to reach the 2nd instar (Clements, 1992): three basic ones (arginine, lysine and histidine) and seven neutral ones (valine, leucine, isoleucine, phenylalanine, tryptophan, threonine and methionine). The positive charge on the three basic amino acids precludes their partition into the lipid bilayer of biomembranes. The other seven essential amino acids could enter lipid bilayers but could not leave them easily. Thus, one would expect to find a subset of transporters for basic amino acids and one for neutral amino acids. The non-essential amino acids (glycine, alanine, tyrosine, cysteine, serine, proline, aspartate and glutamate) and amides (asparagine and glutamine) are

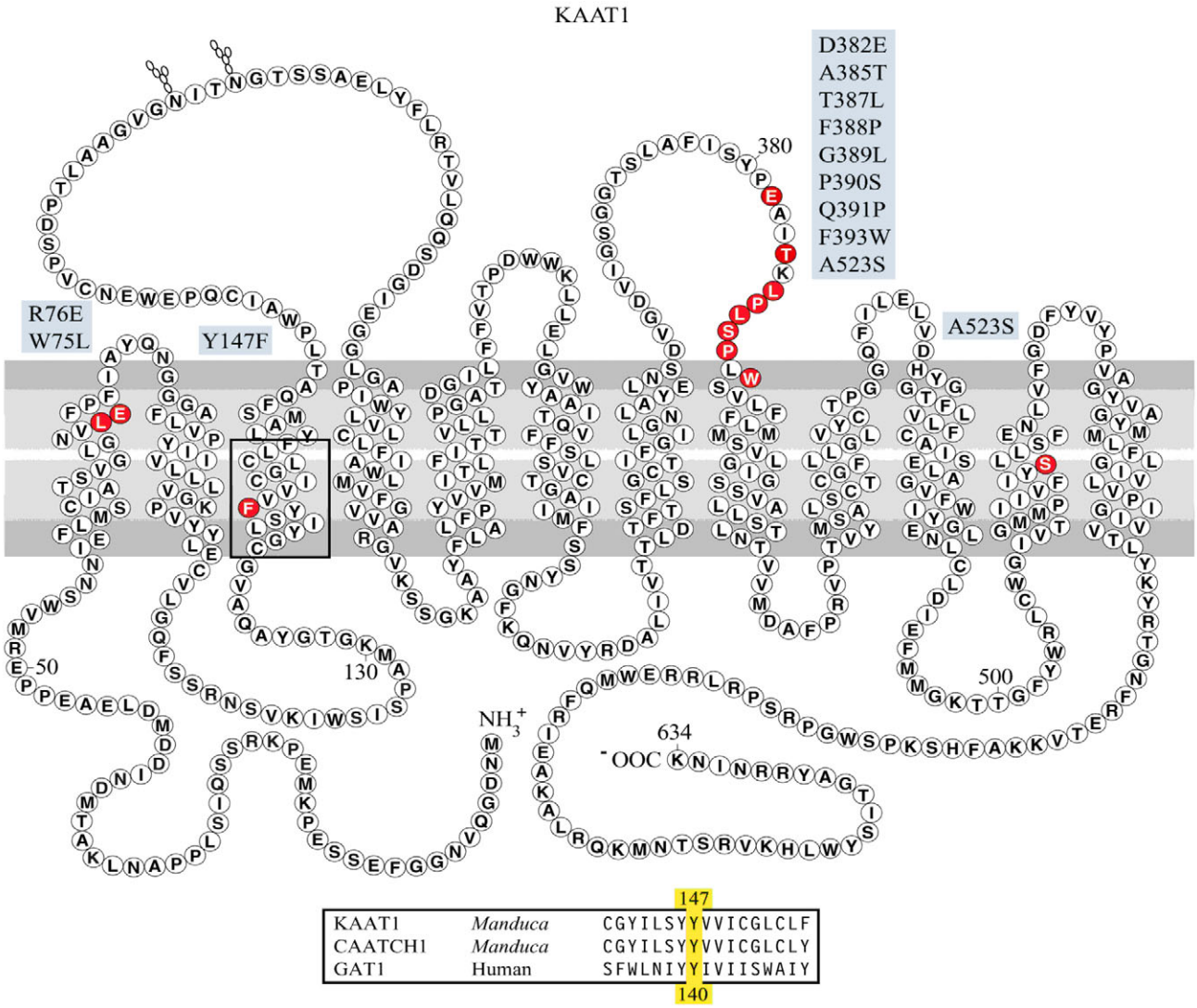

Fig. 1. Predicted secondary structure of K^+ amino acid transporter 1 (KAAT1) polypeptide, showing the location of mutations that were made to investigate function (blue, mutation; red, location) and tyrosine 147 (yellow Y in lower box) that is conserved among related transporters; from Liu et al. (Liu et al., 2003).

synthesized within cells. Nevertheless, specific transporters for non-essential amino acids, which are important for neurotransmission and many metabolic pathways, have evolved (Boudko et al., 2005b).

The major energizer of solute transport in the midgut epithelium of mosquito larvae is ATP hydrolysis by the proton-translocating (H^+) V-ATPase. This primary pump is highly expressed in GC and PMG epithelial cells of *Ae. aegypti* larvae where it is localized in the apical plasma membranes (Patrick et al., 2006; Zhuang et al., 1999). It is thought to drive ion-coupled, electrophoretic amino acid uptake from ectoperitrophic space to cells. The Na^+/K^+-ATPase also plays a prominent role in larval mosquito membrane energization (Patrick et al., 2006); the default condition seems to be that H^+ V-ATPases energize apical membranes and Na^+/K^+-ATPases energize basal membranes. However, H^+ V-ATPase is in the basal membrane in larval *Ae. aegypti* anterior midgut (AMG) (Zhuang et al., 1999) and Na^+/K^+-ATPase is in the apical membrane (Patrick et al., 2006), as they are in *An. gambiae* larvae (Okech et al., 2008a).

Cloning in the pre-genomic era: *Ms*KAAT1, *Ms*CAATCH1 and *Ae*AAT1i

Although Guastella and colleagues had cloned the γ-amino butyric acid (GABA) transporter (GAT1) in 1990 (Guastella et al., 1990), it was not until 1998 that K^+ amino acid transporter 1 (KAAT1) (Fig. 1), the first metazoan, nutrient α-amino acid transporter, was cloned and characterized (Castagna et al., 1998). KAAT1 was cloned by RNA size-fractionation/expression in *Xenopus laevis* oocytes that had been injected with cRNA from *Manduca sexta* midgut. KAAT1 has 634 amino acid residues with 12 putative membrane spanning domains (Fig. 1) and shows a low level of identity with members of the Na^+- and Cl^--coupled neurotransmitter transporter (NTT) family. To identify the amino acid binding sites several mutations that had been identified in the GABA transporter (GAT1) were made (Fig. 1, blue shading). Mutating tyrosine 147 to phenylalanine (yellow shading) increased labeled leucine uptake by *Xenopus* oocytes in Na^+ buffer by seven-fold whereas mutation of the equivalent site, Y140 in GAT1, led to complete loss of activity (Liu et al., 2003). Further mutations of amino acid residues in *M. sexta* K^+ amino acid transporter 1 (*Ms*KAAT1) have been analyzed by the Italian workers (references listed below).

In situ hybridization revealed that KAAT1 cRNA is transcribed in labial glands and in absorptive columnar cells of the caterpillar midgut where K^+ is the principal cation. Its kinetic properties are similar to those of neutral amino acid transport systems in BBMV from this caterpillar (Wolfersberger, 2000). The cation dependency, amino acid uptake activity and kinetic properties of KAAT1 have subsequently been studied by many workers (e.g. Bossi et al., 1999a; Bossi et al., 1999b; Bossi et al., 2000; Peres and Bossi, 2000; Vincenti et al., 2000; Castagna et al., 2002; Liu et al., 2003).

CAATCH1 is a substrate-gated ion channel and an ion-gated transporter

A second amino acid transporter, cation anion activated amino acid transporter channel (CAATCH1) was cloned by Feldman and colleagues from *M. sexta* (Feldman et al., 2000). When expressed in *Xenopus* oocytes in the presence of L-proline, L-threonine or L-methionine, CAATCH1 exhibited an inverted U-shaped current–voltage relationship, which is characteristic of the ion-gated transporters of dopamine, serotonin and norepinephrine in the presence of cocaine or antidepressants. The sharply increasing current with increasingly negative voltages shows that CAATCH1 is electrophoretic (Feldman et al., 2000). However, unlike other sodium neurotransmitter symporter family (SNF) transporters, CAATCH1 activity is independent of Cl^-. Its substrate-associated currents resemble those of KAAT1 in that it is an alkali cation-activated, voltage-dependent, nutrient amino acid carrier. However, unlike KAAT1, CAATCH1 possesses a methionine-inhibitable leakage current and has a variable narrow substrate selectivity, preferring threonine in the presence of K^+ but proline in the presence of Na^+. CAATCH1 is pH dependent, its activity increasing to at least pH 9.5 in oocyte membranes. The activity of CAATCH1 heterologously expressed in *Xenopus* oocytes is similar to that *in vivo* where the transapical voltage is –240 mV and the pH is >10. Soragna and colleagues studied differences in amplitude, kinetics and voltage dependence of transport-associated currents between KAAT1 and CAATCH1 (Soragna et al., 2004). They constructed four chimeric proteins between the two transporters, expressed them in *Xenopus* oocytes and analyzed them by two-electrode voltage clamp and tracer uptake experiments. Analysis of the data from the four chimeras revealed that only central membrane domains were responsible for selectivity. Quick and Stevens studied the relationship between amino acid transport and amino acid-gated ion fluxes (pre-steady state transient and steady state currents) mediated by CAATCH1 expressed in *Xenopus*

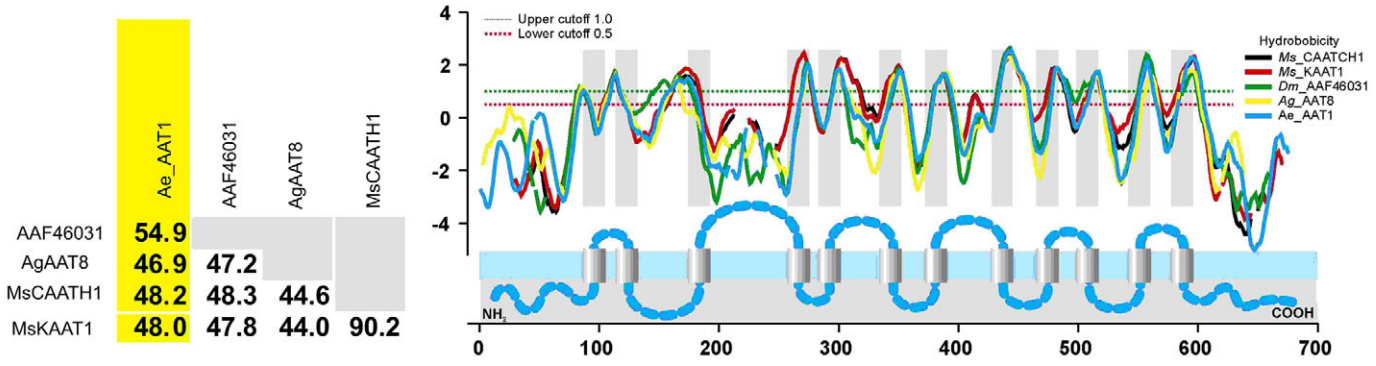

Fig. 2. Characteristics of *Aedes aegypti* amino acid transporter 1 (*Ae*AAT1i). Reciprocal amino acid sequence identity matrix (left); hydropathy plot and predicted transmembrane topology (right); TopPed II parameters were: GES-hydrophobicity scales: 1.0 upper and 0.5 lower cutoffs; 10 and 5; core and wage window sizes, 60; critical loop length, 2 for critical transmembrane spacer. Hydropathy values (vertical scale) and distribution of transmembrane spanning domain (insert) are aligned relative to amino acid position (horizontal scale). (Modified from Boudko et al., 2005a.)

oocytes (Quick and Stevens, 2001). Simultaneous tracer flux and electrical current measurements showed that cation and amino acid transport events are thermodynamically uncoupled. Quick and Stevens concluded that CAATCH1 is a multi-function membrane protein, which acts primarily as an amino acid-gated alkali cation channel but it also mediates thermodynamically uncoupled amino acid uptake (Quick and Stevens, 2001).

*Ae*AAT1 was the first mosquito nutrient amino acid transporter to be was cloned

Amino acid transporter 1 (*Ae*AAT1i) was cloned by a PCR procedure from a PMG cDNA collection of *Ae. aegypti* larvae (Fig. 2). It is a 678 residue polypeptide with high amino acid sequence identity to *Ms*KAAT1, *M. sexta* cation anion activated amino acid transporter channel (*Ms*CAATCH1), *Ag*NAT8

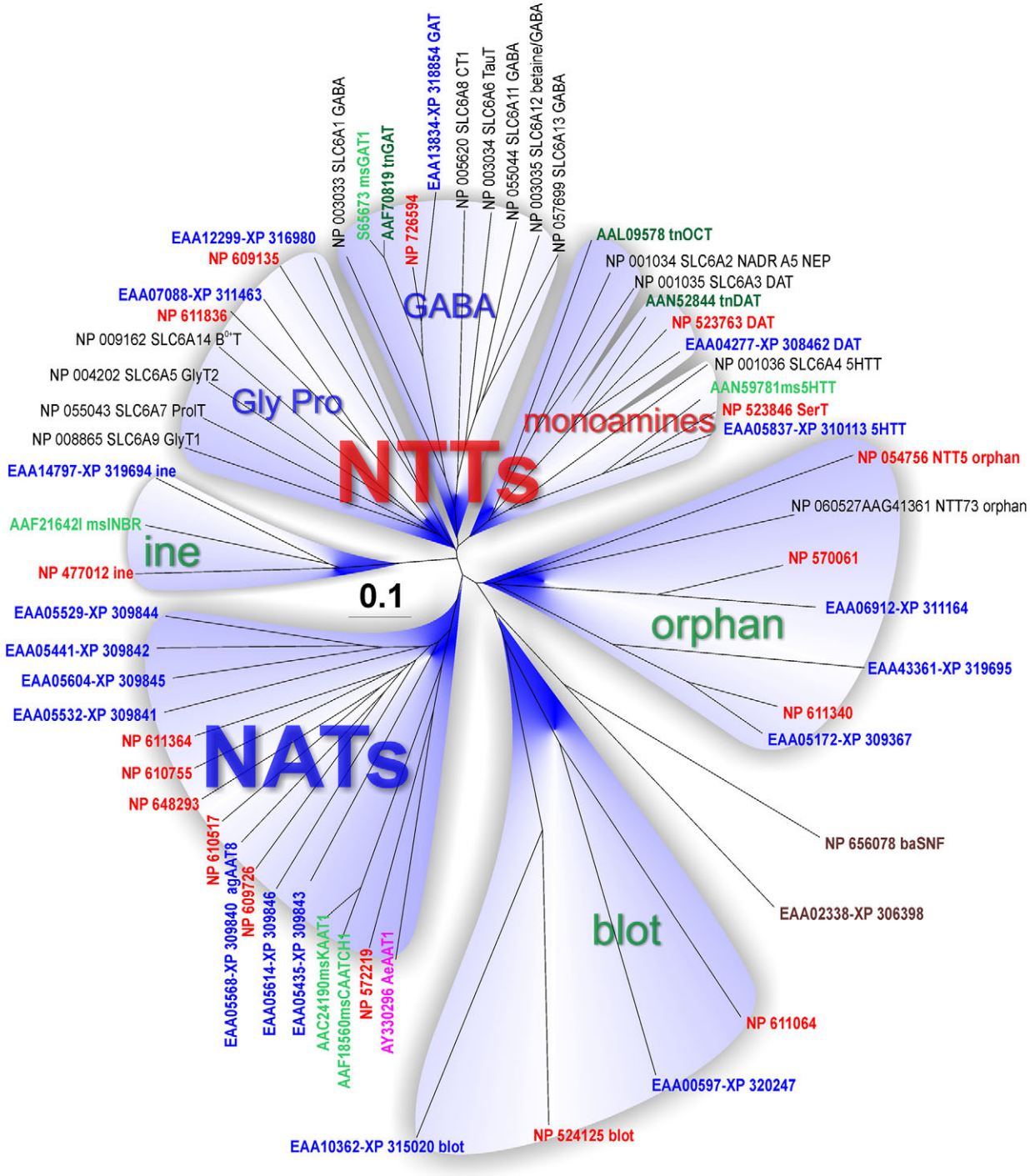

Fig. 3. Phylogram of sodium neurotransmitter symporter family (SNF) showing the relationship of nutrient amino acid transporters (NATs) to neurotransmitter transporters (NTTS). NATs are Na^+-coupled amino acid$^{\pm}$ (neutral amino acid) symporters. Accession numbers for the seven *Anopheles gambiae* NATs are shown in blue font (Boudko et al., 2005b).

(Meleshkevitch et al., 2006) and a putative gene product from *D. melanogaster* (Fig. 2, left). *Ae*AAT1 has 12 transmembrane sectors at positions similar to those in *Ms*KAAT1 and *Ms*CAATCH1 (Fig. 2, right). When heterologously expressed in *Xenopus* oocytes, the amino acid-induced inward currents were Na^+ dependent but were K^+ and Cl^- independent. Nevertheless, K^+ and Cl^- modified the response kinetics and *I*/*V* dependency of the transporter. The amplitude of the ligand-induced currents depended upon pH and transmembrane voltage. The transport showed saturable kinetics for both Na^+ and amino acid with apparent K_m of 0.45±0.05 and 37.65±2.12 mmol l^{-1}, and Hill coefficients 1.04±0.08 and 2.05±0.23 for phenylalanine and Na^+, respectively. These data suggest that *Ae*AAT1i is an electrophoretic transporter with stoichiometry 1(amino acid):2(Na^+).

Cloning in the post-genomic era

NTTs and NATs from genomic mosquitoes

The SNF (also known as SLC6 or HUGO) is one of the largest, most ancient and most diverse families of secondary transporters. Members of its neurotransmitter transporter (NTT) subfamily mediate absorption of neurotransmitters: dopamine, norepinephrine, epinephrine, octopamine, serotonin, GABA; putative neuromodulators (glycine and proline) intracellular osmolytes (taurine and betaine) intracellular energy substrates

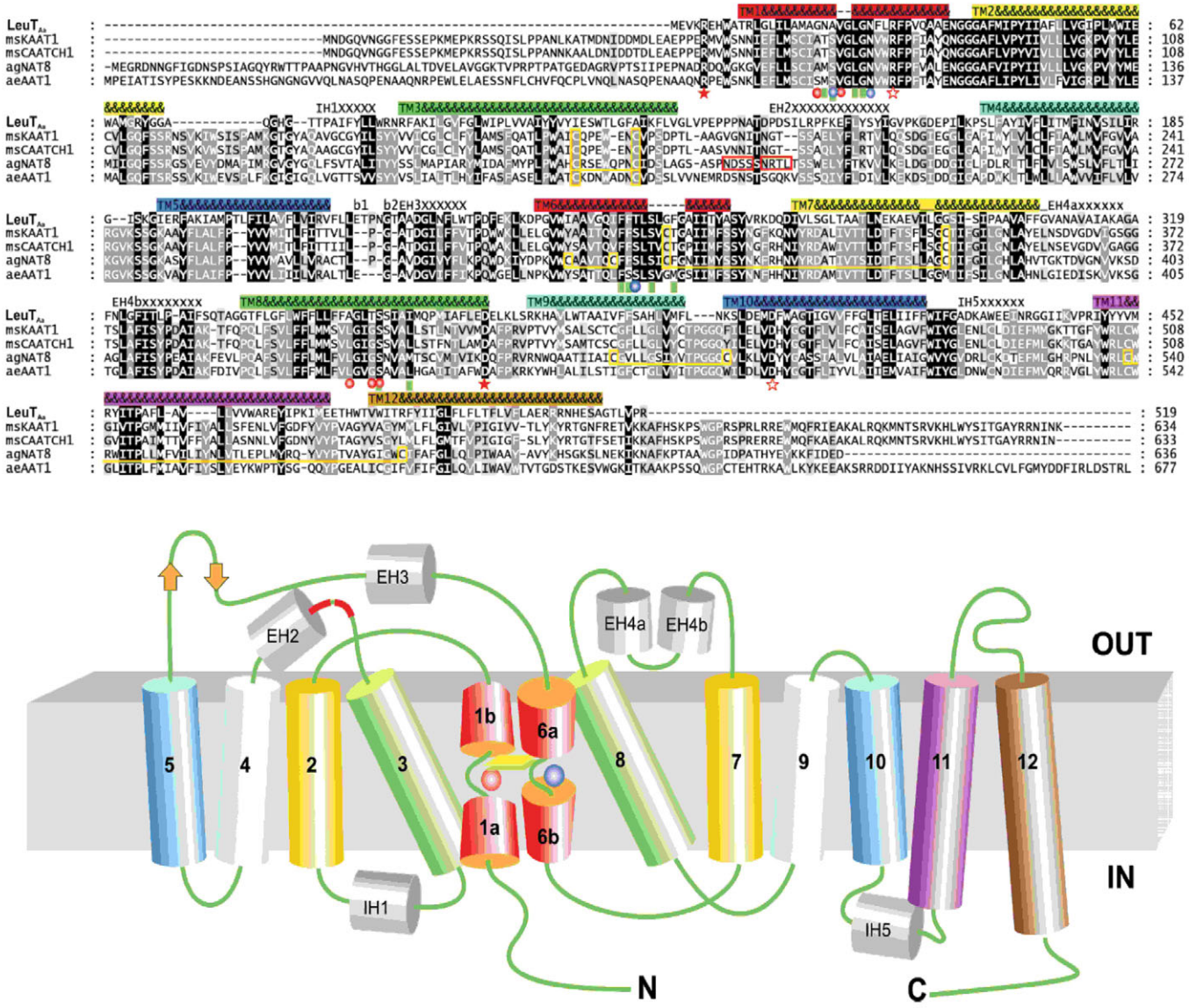

Fig. 4. Alignment and reconstruction of *Anopheles gambiae* nutrient amino acid transporter 8 (*Ag*NAT8) structure: (A) sequence/structure alignment of characterized insect transporters relative to the first crystallized bacterial nutrient amino acid transporters (NAT) from *A. aeolicus*, LeuTAa (Yamashita et al., 2005). (B) 2-D structure of *Ag*NAT8 based on structural homology with the LeuTAa protein sequence. NCBI Accession no.: LeuTAa, NP_214423 (PDB no., 2A65); *Manduca sexta* K^+ amino acid transporter 1 (*Ms*KAAT1), AAC24190; *M. sexta* cation amino acid transporter channel 1 (*Ms*CAATCH1), AAF18560; *Aedes aegypti* amino acid transporter 1 (*Ae*AAT1), AAR08269; *Ag*NAT8, AAN40409. Filled and open stars represent putative cationic gates at extra- and intracellular interfaces, respectively. Squares indicate putative substrate binding sites; red and blue spheres outline sites that interact with the first and second sodium ion, respectively. Red and yellow boxes show putative glycosylation motifs and disulfide bridges, respectively. The 12 transmembrane domains are numbered 1–12; from Meleshkevitch et al. (Meleshkevitch et al., 2006).

(creatine and proline) and a number of 'orphan' proteins. Several insect NTTs have been cloned, expressed in *Xenopus* oocytes and functionally characterized. The GABA transporter was cloned and localized in *M. sexta* (Mbungu et al., 1995), glutamate/aspartate transporters have been cloned from mosquito (Umesh et al., 2003) and *Trichoplusia ni* (Gao et al., 1999) central nervous systems. High-affinity, Na^+-dependent glutamate transporters have been cloned from *T. ni* (Gardiner et al., 2002), from cockroach *Diploptera punctata* [excitatory amino acid transporter (EAAT1)] (Donly et al., 2000) and from *Drosophila* (Seal et al., 1998); they are similar to vertebrate neurotransmitter transporters and are predominantly localized in the brain (Donly et al., 1997).

The NAT subfamily is the largest subdivision of the SNF (Fig. 3). There are seven members of the NATs population in the African malaria mosquito, *Anopheles gambiae* [blue font in Boudko et al. (Boudko et al., 2005b)]. Two of its members have been cloned and characterized – *Ag*NAT8 was published two years ago (Meleshkevitch et al., 2006) and a *Ag*NAT6 manuscript is currently under editorial review. The synergistic localization of the two transporters was published recently (Okech et al., 2008b).

*Ag*NAT8 was cloned from *An. gambiae* (Meleshkevitch et al., 2006) (Fig. 4). It performs Na^+-coupled nutrient absorption, preferring phenylalanine and its derivatives, tyrosine and L-DOPA (3,4-dihydroxy-L-phenylalanine). It is transcribed at specific sites in the central and peripheral neurons including visual-, chemo- and mechano-sensory afferents. It is widely transcribed in the alimentary canal where it displays alternative, apical *vs* basal docking in absorptive *vs* secretory regions. Several putative phosphorylation sites and other post-translational modification motifs are present in external loops and transmembrane domains (Fig. 4).

*Ag*NAT8 is electrophoretic, which is clear from the large inward current (Fig. 5) and its dependence on voltage as seen in the *I*/*V* plots (below). The reversible stimulation of current to >300 nA by phenylalanine in the presence of Na^+ confirms that *Ag*NAT8 is a Na^+:amino acid symporter. The current is barely affected by Cl^- removal but, as in *Ae*AAT1i, it is abolished when Na^+ is replaced by either K^+ or Li^+. The inability of K^+ to sustain inward currents in mosquito NATs was unexpected in light of the brush-border membrane studies on caterpillars (e.g. Giordana et al., 1998; Giordana et al., 1989; Hennigan and Wolfersberger, 1989). This difference between mosquito NATS and their caterpillar cousin, KAAT1, which prefers K^+ to Na^+, may reflect the difference between the constantly high K^+/low Na^+ leafy diet of caterpillars and the varied diet of mosquito larvae.

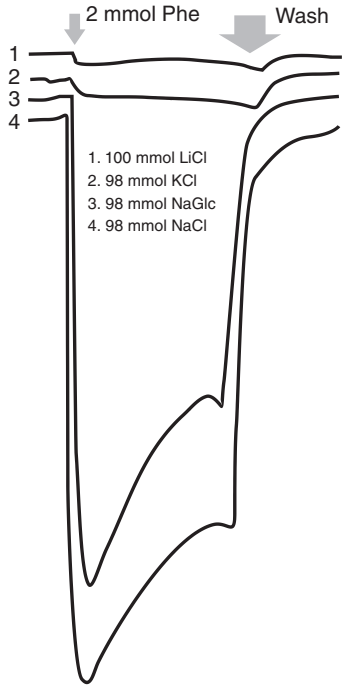

Fig. 5. When expressed in *Xenopus* oocytes *Anopheles gambiae* nutrient amino acid transporter 8 (*Ag*NAT8) exhibits large inward currents showing that it is electrophoretic (Meleshkevitch et al., 2006).

Synergy between *Ag*NAT8 and *Ag*NAT6

Most recently, another new member of the NAT-SLC6 (solute carrier clade of amino acid transporters) group of the NSF has been cloned and designated, *Ag*NAT6 (*Anopheles gambiae* nutrient amino acid transporter 6) (E. A. Meleshkevitch, M. Robinson, L. B. Popova, M. M. Miller, W.R.H. and D.Y.B., unpublished data). This new transporter from *An. gambiae* was localized by *in situ* hybridization and by immunohistochemistry (Fig. 6) (Okech et al., 2008b). *Ag*NAT6 is extensively transcribed throughout the alimentary canal where its localization implies that it functions both in the primary absorption and subsequent secretion of these aromatic amino acids. It is also transcribed in specific neuronal structures, including the neuropile of ventral ganglia and sensory afferents.

The relative expression and distribution of the two aromatic NATs were examined with transporter-specific antibodies in mosquito larval alimentary canal (Okech et al., 2008b). The immunolabeling showed a strong correlation between functional expression and localization of both *Ag*NAT6 and *Ag*NAT8 in the plasma membrane of frog eggs (data not shown). Both transporters exhibited elevated expression in specific regions of the larval alimentary canal of *An. gambiae*, including salivary glands, cardia, GC, PMG and Malpighian tubules (Fig. 6). Differences in relative expression densities and spatial distribution of the transporters were prominent in virtually all of these regions suggesting unique profiles of aromatic amino acid absorption. For the first time reversal of location of a transporter between apical and basal membranes was identified in posterior and anterior epithelial domains corresponding with secretory and absorptive epithelial functions, respectively.

Okech and colleagues argued that the results suggest functional synergy between substrate-specific *Ag*NAT6 and *Ag*NAT8 in intracellular absorption of aromatic amino acids (Okech et al., 2008b). More broadly, they suggest that the specific selectivity, regional expression and polarized membrane docking of NATs represent key adaptive traits shaping functional patterns of essential amino acid absorption in the metazoan alimentary canal. Like all of the other cloned NATs, *Ag*NAT6 is electrogenic based on large inward currents and the voltage dependence of its *I*/*V* plots (E. A. Meleshkevitch, M. Robinson, L. B. Popova, M. M. Miller, W.R.H. and D.Y.B., unpublished data).

Electrical characterization of amino acid transporters

When expressed heterologously in *Xenopus* oocytes, KAAT1 mediated electrophoretic transport of neutral amino acids (Fig. 7). Moreover, uptake was Cl^- dependent. K^+, Na^+ and, to a lesser extent, Li^+ were accepted as cotransported ions. The K^+/Na^+ selectivity increased with oocyte hyperpolarization as it does upon hyperpolarization of isolated midguts. The conductance-

increase accelerated at voltages >–70 mV suggesting that KAAT1 may function as a channel at very negative potentials. All of the NATs that have been cloned to date are electrophoretic as is clear from the *I*/*V* plots (Fig. 7). The currents are always inward. *Ae*AAT1 is unlike other NATs in that there is no region of constant conductance. KAAT1 and *Ag*NAT8 show surprisingly large inward currents. The current increases in an accelerating manner with very negative voltages. These increases are not likely to be simple non-selective leaks because they appear in all four *I*/*V* plots of Fig. 7.

The importance of being electrogenic

The Na^+ gradient, $Na^+_{outside}/Na^+_{inside}$ across the apical membrane of larval *An. gambiae* midgut is approximately 25 mmol l^{-1}/10 mmol l^{-1}; the size of the H^+ and amino acid gradients are unknown but they are assumed to be too small to drive the amino acid uptake. However, the –60 to –120 mV V_m [outside positive (D.Y.B. and W.R.H., unpublished data)] is equivalent to a 10- to 100-fold concentration gradient. Na^+ concentration gradients are invariably discussed as energizers of epithelial membranes but the electrical gradients are often ignored. At scientific meetings, one

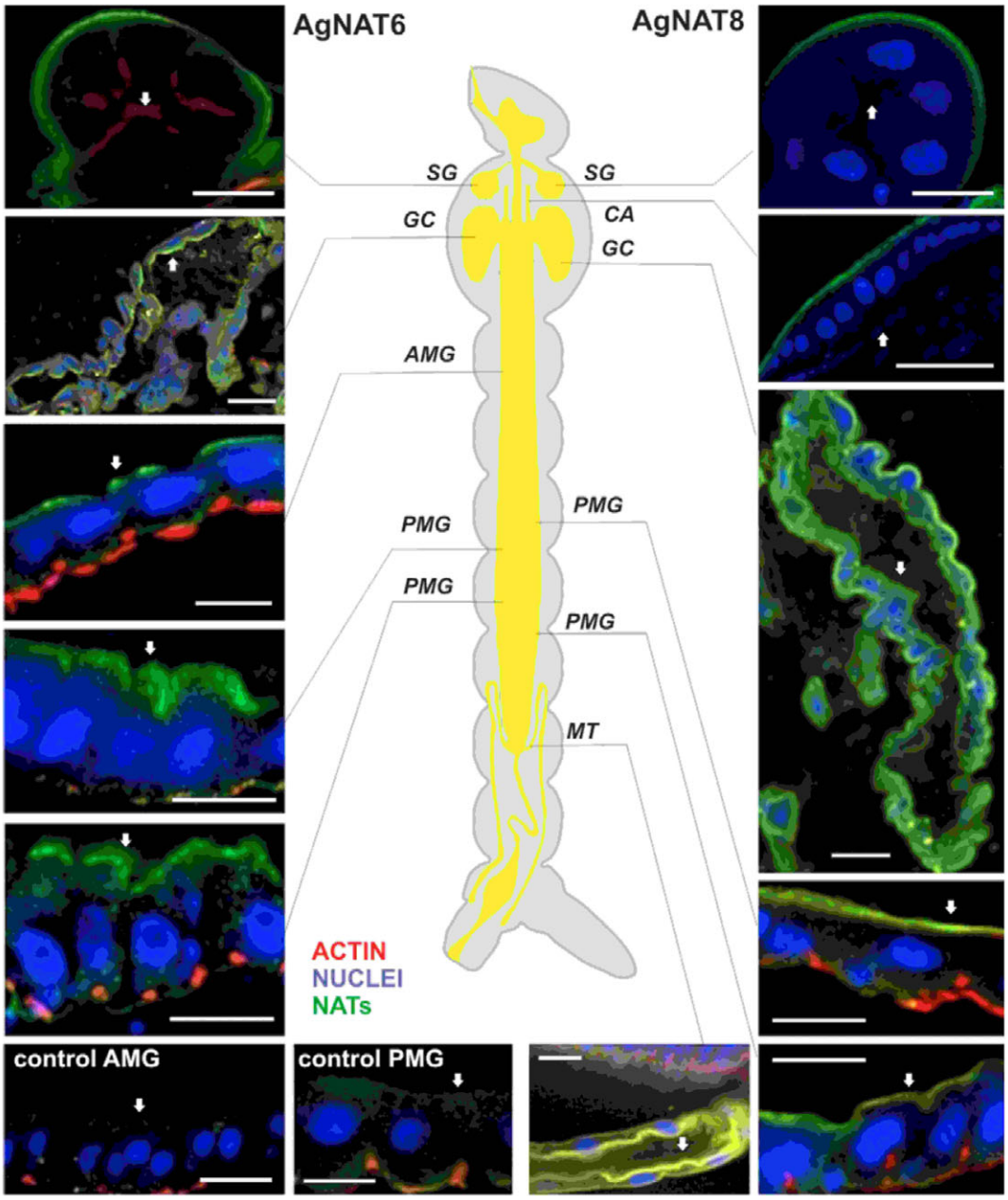

Fig. 6. Immunolabeling of *Anopheles gambiae* nutrient amino acid transporters (*Ag*NATs) in frozen sections of the larval alimentary with epitope-specific purified antibodies (green channel) along with actin (TRITCPhalloidin, red channel) and nuclei (DRAQ-5, blue channel) viewed by confocal microscopy. Actin and nuclei were not visualized in a few sections to improve overall clarity. The red channel generally represents actin in the muscular envelope around the alimentary canal and corresponds to the location of the basal membrane except in salivary glands and Malpighian tubules where actin reveals the location of microvilli on the apical membrane. The location of the apical membrane is indicated by white arrows. Approximate positions of individual sections are shown at the insert diagram. Abbreviations are: SG, salivary gland; CA, cardia; GC, gastric caeca; AMG, anterior midgut; PMG, posterior midgut; MT, Malpighian tubes. Control sections of the AMG (control AMG) and PMG (control PMG) incubated with pre-bleed serum shown at the left bottom corner insert. Scale bar, 50 μm (Okech et al., 2008b).

often sees diagrams with H^+ V-ATPases located on a cell membrane without any indication of the magnitude or polarity of the voltage. Yet it is unlikely that metazoans would be the only taxa that rely solely on Na^+ gradients and fail to use voltage gradients for secondary ion transport, especially because all genomic metazoans possess primary electrogenic H^+ V-ATPases and numerous electrophoretic secondary transporters. The electrically coupled NHE_{VNAT} pair can scarcely avoid playing a significant role in mosquito ion regulation as it is widely acknowledged to do in amino acid uptake.

It is widely accepted that the voltage generated by H^+ V-ATPases drives the symport of Na^+ stoichiometrically coupled to an amino acid into alimentary canal epithelial cells of many insects. As noted above, the amino acid uptake role of the symporters is emphasized with little attention paid to its Na^+ uptake role. Nevertheless, Na^+ uptake has four important consequences. (1) Removing Na^+ from the lumen allows the H^+ that is sequestered on the luminal face of the apical membrane by the H^+ V-ATPase to enter the bulk phase and de-alkalinize the lumen in the PMG. (2) Adding H^+ to the lumen and Na^+ to the cells removes metabolic acid from cells, complementing classical NHEs in this respect. (3) Removing Na^+ from the lumen would lower its concentration there and amino acid symport would stop. (4) Adding H^+ to the lumen and removing Na^+ would continue to acidify it. As the lumen and cells must be in an ionic steady state, these results imply that an additional transporter must be present. The postulated transporter should have the same orientation as the electrophoretic bacterial NHA, which uses the outside positive voltage generated by the electron transport system to drive $2H^+$ into cells and Na^+ out. Because *Ag*NHA1 is located in the same membranes as the H^+ V-ATPase and *Ag*NAT8 (see VAN in Fig. 8) (Rheault et al., 2007; Okech et al., 2008a) and because it is a distant relative of the NHAs in alkalophilic transporters (Brett et al., 2005), it is worth considering as a candidate for this role.

Speculation regarding V-ATPase, NAT, NHA (VAN) as a localized homeostatic trio

The preceding discussion has been based on data published in refereed journals. Here, we speculate on the implications of the data for ionic homeostasis. To make the case that *Ag*NHA1 is a candidate for the recycling role, consider alkalophilic bacteria as models. These distant relatives, like *Ag*NHA1, face a highly alkaline environment (Krulwich et al., 1994). The bacterial antiporter expressed in *E. coli* as *Ec*NhA1 (*E. coli* Na^+/H^+ antiporter 1) is the most well-known cation exchanger; its crystal structure and mechanism of action have been explored in a series of brilliant studies (Hunte et al., 2005; Padan et al., 2005; Padan et al., 2009). Bacterial NHAs use the V_m generated by the electron transport system to drive $1Na^+$ out of cells and $2H^+$ into them (Taglicht et al., 1993); thus, they act in the opposite direction from NHEs or NHE_{VNAT}s and in the direction required of our candidate NHA. If *Ag*NHA1 functions like bacterial NHAs, it would fulfil the H^+ and Na^+ recycling role mentioned above.

The H^+ V-ATPase, *Ag*NAT8 and *Ag*NHA1 trio (VAN) is localized in the apical membrane of the epithelial cells in PMG of *An. gambiae* larvae (Fig. 8) (Rheault et al., 2007; Okech et al., 2008a) where the luminal pH is much lower than in anterior midgut (AMG). It is clear that the electrogenic H^+ V-ATPase drives H^+

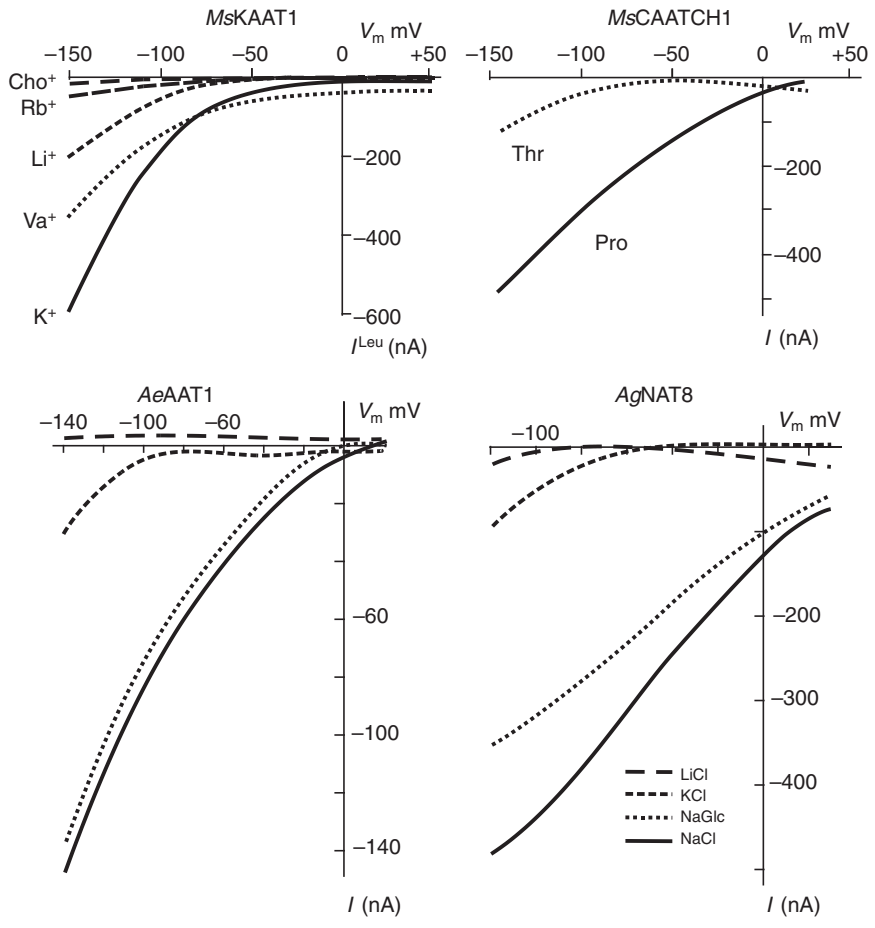

Fig. 7. Current/voltage (*I*/*V*) relationships of four (Na^+ or K^+):amino acid$^{\pm}$ symporters (NATs). In all four cases the current is voltage dependent. In *Manduca sexta* K^+ amino acid transporter 1(*Ms*KAAT1) and *Aedes aegypti* amino acid transporter 1 (*Ae*AAT1) the conductance is linear from 0 to ~–60 mV then increases at voltages more negative than –70 mV. These results demonstrate that all four NATs are voltage driven (electrophoretic): (A) *Manduca sexta* K^+ amino acid transporter 1 (*Ms*KAAT1) (Castagna et al., 1998): (B) *M. sexta* cation amino acid transporter channel 1 (*Ms*CAATCH1) (Feldman et al., 2000); (C) *Ae. aegypti* amino acid transporter 1 (*Ae*AAT1) (Boudko et al., 2005a): (D) *An. gambiae* nutrient amino acid transporter 8 (*Ag*NAT8) (Meleshkevitch et al., 2006).

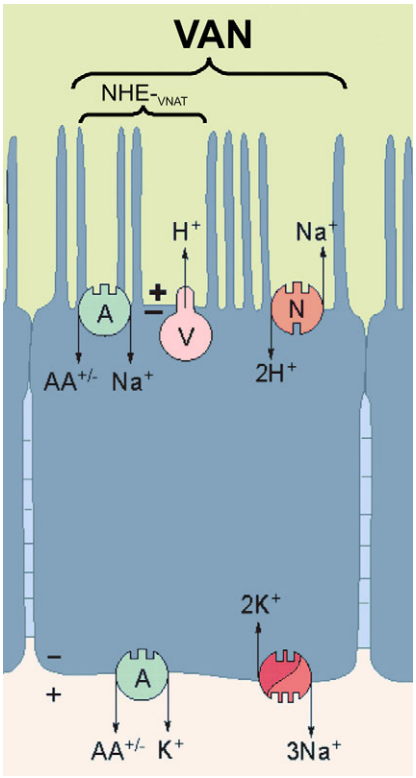

Fig. 8. An H^+ V-ATPase (V) is electrically coupled by the membrane potential (V_m) to AgNAT8, a Na^+:amino acid transporter (NAT; N) in the apical plasma membrane of posterior midgut (PMG) constituting an NHE_{VNAT}. *Ag*NHA1, a Na^+/nH^+ Antiporter (NHA; A), is also present on the apical membrane. A Na^+/K^+ ATPase is present in the basal membrane along with *Anopheles gambiae* nutrient amino acid transporter 6 (*Ag*NAT6) (modified from Okech et al., 2008a; Okech et al., 2008b).

across the membrane's lipid bilayer and hyperpolarizes it. It is also clear that the electrophoretic NHE_{VNAT8} replaces lumen Na^+ by H^+ and de-alkalinizes it. As NATs constitute a major pathway for amino acid absorption, similar NHE_{VNAT} activity is expected to be widespread in insects if not in other metazoan organisms. Furthermore, it is tempting to speculate that in larval GC and PMG the outside positive voltage drives Na^+ back out of the cells coupled to $2H^+$ entry *via* the *Ag*NHA1 that is located there (Okech et al., 2008a) completing the VAV trio. If these speculated properties of *Ag*NHA1 are correct, then the presence of all three proteins in the apical membrane of both GC and PMG cells would provide an integrated mechanism for amino acid uptake, metabolic acid expulsion, lumen alkalinization and de-alkalinization and Na^+ recycling. Whether the midgut lumen is alkalinized or de-alkalinized would depend on the relative activities of the NAT and NHA components. NHAs (Na^+ out, $2H^+$ in) would dominate in GC and the lumen would be alkalinized; NATs (Na^+ in, H^+ out) would dominate in PMG and the lumen would be de-alkalinized. The primary energy source for these integrated mechanisms would be the trans-apical membrane potential generated from ATP hydrolysis via the H^+ V-ATPase. Its value of ~ –60 to –120 mV (≡10-, 100-fold Δ concentration) would be sufficient to energize both electrophoretic NHAs and NATs whereas Δ concentrations of Na^+, H^+ and amino acid$^{\pm}$ (neutral amino acid) are all much too small for this purpose.

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LIST OF ABBREVIATIONS

*Ae*AAT1	*Aedes aegypti* amino acid transporter 1 (also known as *Ae*AAT1i in the original publication and GenBank. Please note that a prefix indicates the genus and species of a donor; the suffix priority of cloning)
*Ae*NHE1	*Aedes aegypti* Na^+/H^+ exchanger 1 [also known as *Ae*NHE8 in Pullikuth et al. (Pullikuth et al., 2006) terminology]
*Ae*NHE2	*Aedes aegypti* Na^+/H^+ exchanger 2 [also known as *Ae*NHE3 in in Pullikuth et al. (Pullikuth et al., 2006) terminology]
*Ag*NAT6	*Anopheles gambiae* nutrient amino acid transporter 6
*Ag*NAT8	*Anopheles gambiae* nutrient amino acid transporter 8
AMG	anterior midgut
BBMV	brush-border membrane vesicle
EAAT	excitatory amino acid transporter
*Ec*NhA1	*E. coli* Na^+/H^+ antiporter 1
GABA	γ-amino butyric acid
GAT1	γ-amino butyric acid transporter
GC	gastric caeca
GCAM	goblet cell apical membrane
*Ms*CAATCH1	*Manduca sexta* cation anion activated amino acid transporter channel 1
*Ms*KAAT1	*Manduca sexta* K^+ amino acid transporter 1
MT	Malpighian tubule
NAT	nutrient amino acid transporter
NHA	Na^+/H^+ antiporter
NHA1	Na^+/H^+ antiporter 1
NHA2	Na^+/H^+ antiporter 2
NHE	Na^+/H^+ exchanger
NTT	neurotransmitter transporter
PMG	posterior midgut
SLC6	solute carrier clade of amino acid transporters
SLC9	solute carrier 9 SLC clade of NHEs and NHAs
SNF	sodium neurotransmitter symporter family
VAN	V-ATPase-NAT-NHA residing in same membrane (a postulated mechanism for regulating pH and Na^+ concentrations in alimentary canal lumen and cells)
V_m	membrane potential (indicates the measured voltage across a membrane)
ΔΨ	electrical potential difference [ΔΨ indicates the theoretical or calculated (thermodynamic) electrical potential of an ionic species]

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