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RESEARCH ARTICLE

Pharmacological characterisation of apical Na^+ and Cl^- transport mechanisms of the anal papillae in the larval mosquito *Aedes aegypti*

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SUMMARY

The anal papillae of freshwater mosquito larvae are important sites of NaCl uptake, thereby acting to offset the dilution of the hemolymph by the dilute habitat. The ion-transport mechanisms in the anal papillae are not well understood. In this study, the scanning ion-selective electrode technique (SIET) was utilized to measure ion fluxes at the anal papillae, and pharmacological inhibitors of ion transport were utilized to identify ion-transport mechanisms. Na^+ uptake by the anal papillae was inhibited by bafilomycin and phenamil but not by HMA. Cl^- uptake was inhibited by methazolamide, SITS and DIDS but not by bafilomycin. H^+ secretion was inhibited by bafilomycin and methazolamide. Ouabain and bumetanide had no effect on NaCl uptake or H^+ secretion. Together, the results suggest that Na^+ uptake at the apical membrane occurs through a Na^+ channel that is driven by a V-type H^+-ATPase and that Cl^- uptake occurs through a Cl^-/HCO_3^- exchanger, with carbonic anhydrase providing H^+ and HCO_3^- to the V-type H^+-ATPase and exchanger, respectively.

Key words: mosquito, anal papillae, ion transport, V-type H^+-ATPase, Na^+/K^+-ATPase.

INTRODUCTION

Larval mosquitoes inhabit freshwater and are thus faced with dilution of their internal body fluids through the passive uptake of water and loss of ions. Observations reveal that larval mosquitoes are well equipped to survive in these dilute conditions through tightly regulating the ion and water content of their hemolymph (Patrick et al., 2001; Patrick et al., 2002a; Patrick et al., 2002b). The function and regulation of major ion- and water-transporting epithelia in the larvae is likely to play a major role in this process. The combined actions of the Malpighian tubules, which secrete ions and water from the hemolymph into their lumen, and the rectum, which reabsorbs ions back into the hemolymph, results in production of a dilute urine (Bradley, 1987). This permits the larvae to conserve ions, while eliminating excess water. The anal papillae are specialized for the uptake of ions from the dilute larval habitat (Stobbart, 1965; Stobbart, 1967; Donini and O'Donnell, 2005), therefore complementing the role of the Malpighian tubules and rectum.

The larvae of the mosquito *Aedes aegypti* have four anal papillae, which developmentally arise from the hindgut, surround the anus and protrude into the external habitat. Each anal papilla consists of an epithelium, a single cell-layer thick, covered by a thin and relatively permeable cuticle (Edwards and Harrison, 1983; Wigglesworth, 1933). The epithelium is extensively tracheated, and the anal papilla lumen is filled with hemolymph and is continuous with the hemocoel (Edwards and Harrison, 1983). The anal papillae are sites of NaCl uptake and H^+ and ammonia secretion (Donini and O'Donnell, 2005). The uptake of both Na^+ and Cl^- measured directly at the anal papillae is saturable, suggesting the existence of carrier-mediated transport (Donini et al., 2007). Ion-substitution studies measuring whole-body NaCl uptake suggested that Na^+ and Cl^- transport is coupled to H^+ and HCO_3^- secretion (Stobbart, 1971). The importance of H^+ secretion for Na^+ uptake was supported by inhibition of total-body Na^+ uptake by the V-type H^+-ATPase (VA) inhibitor bafilomycin (Patrick et al., 2002b). By contrast, the carbonic anhydrase inhibitor acetazolamide did not alter whole-body Na^+ or Cl^- uptake (Patrick et al., 2002b). As carbonic anhydrase would provide HCO_3^- for Cl^-/HCO_3^- exchange, the latter result does not support the Cl^-/HCO_3^- transport mechanism model. Instead, as bafilomycin potentiated whole-body Cl^- uptake, a Cl^-/H^+ co-transporter mechanism was postulated (Patrick et al., 2002b). The expression of the primary ion-motive pumps Na^+/K^+-ATPase (NKA) and V-type H^+-ATPase has been localized to the basal (lumen facing) and apical (exterior facing) membranes of the anal papilla epithelium, respectively, further reinforcing the link between Na^+ uptake and H^+ secretion (Patrick et al., 2006). The nature of the Na^+ and Cl^- transporters remains elusive. Neither amiloride (inhibitor of Na^+ channels and Na^+/H^+ exchangers) nor bumetanide [inhibitor of $Na^+/K^+/Cl^-$ co-transporters (NKCCs)] affected whole-body Na^+ and Cl^- uptake in whole larvae (Patrick et al., 2002b).

This study set out to characterize the ion-transport mechanisms in the anal papillae of larval *Aedes aegypti*. The scanning ion-selective electrode technique (SIET) was used to measure ion fluxes directly at the anal papillae, and this was combined with application of specific ion-transport inhibitors. The advantage of this approach is that fluxes can be entirely attributed to transport at the anal papillae rather than the whole-body fluxes that are the result of transport at all of the ion- and water-transporting tissues of the larva. The study provides a functional basis for identifying ion transporters that are required for NaCl uptake at the anal papillae.

MATERIALS AND METHODS

Mosquitoes

A laboratory colony of *Aedes aegypti* (Linnaeus) was maintained in the Department of Biology, York University (Toronto, Canada)

at 24°C, 50% humidity and a 12 h:12 h light–dark cycle. The larvae were reared in 5 l capacity plastic tubs containing 2.5 l of distilled water. Larvae were fed daily with 1 ml of a liver powder and yeast solution, and the water was replaced weekly. Studies were conducted on fourth-instar larvae.

Construction of ion-selective microelectrodes and scanning ion-selective electrode technique

Ion-selective microelectrodes (ISMEs) were fabricated utilizing a protocol adapted from Smith et al. and explained in detail by Nguyen and Donini (Smith et al., 1999; Nguyen and Donini, 2010). In brief the following ionophore cocktails and backfill electrolyte solutions (in parentheses) were used: Na^+ ionophore II cocktail A (100 mmol l^{-1} NaCl); Cl^- ionophore I cocktail A (1 mol l^{-1} NaCl); Cl^- ionophore II/TDMACL/o-NPOE (100 mmol l^{-1} NaCl, Messerli et al., 2008) and H^+ ionophore I cocktail B (100 mmol l^{-1} NaCl and 100 mmol l^{-1} sodium citrate, pH 6.0). Na^+ and Cl^- ISMEs were calibrated in 0.1 mmol l^{-1} and 1 mmol l^{-1} NaCl. H^+ ISMEs were calibrated in 1 mmol l^{-1} NaCl containing 1 mmol l^{-1} Hepes adjusted to pH values of 7, 8 and 9 with NaOH or HCl. The ISME slopes were as follows (mean±s.e.m., N): Na^+ (55.78±0.82, 21), Cl^- (57.77±0.52, 62) and H^+ (54.71±0.91, 22).

The SIET methodology is described in detail elsewhere (see Nguyen and Donini, 2010; Rheault and O'Donnell, 2001; Rheault and O'Donnell, 2004). ISMEs were connected to a headstage with an Ag–AgCl wire electrode holder (World Precision Instruments) and the headstage was connected to an ion polarographic amplifier (IPA-2, Applicable Electronics, Forestdale, MA, USA). The reference electrode was a 3% agar in 3 mol l^{-1} KCl bridge connected to the headstage through an Ag–AgCl half-cell (WPI). For each single-point measurement of an ion concentration gradient, the ISME was positioned 5–10 μm from the papilla surface and a voltage was recorded. The ISME was moved between 20 and 100 μm away from the surface of the papilla and a second voltage was recorded after a 4–10 s period of time during which no recording took place. Control measurements to account for the mechanical disturbances in the ion gradients that arise from the movement of the microelectrodes were taken 2–5 mm away from the surface of the anal papillae. Automated scanning electrode technique (ASET) Version 2.0 software (Science Wares, East Falmouth, MA, USA) was used to position the ISMEs and record voltage measurements. This sampling protocol was previously established and utilized for measuring ion gradients at the anal papillae of mosquitoes and midges (see Donini and O'Donnell, 2005; Nguyen and Donini, 2010).

Calculation of ion fluxes

Ion concentration gradients were calculated from the voltage gradient measurements provided by the ASET software with the following equation (Donini and O'Donnell, 2005):

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

where ΔC is the concentration gradient between the two points measured in μmol l^{-1} cm^{-3}; C_B is the background ion concentration, calculated as the average of the concentration at each point measured in μmol l^{-1}; ΔV is the voltage gradient obtained from ASET in μV; and S is the slope of the electrode. Ion flux was calculated with the resulting concentration gradient using Fick's law of diffusion as follows:

$$J_I = D_I(\Delta C) / \Delta x , \quad (2)$$

where J_I is the net flux of the ion in pmol cm^{-2} s^{-1}; D_I is the diffusion coefficient of the ion (1.55×10^{-5} cm^2 s^{-1} for Na^+ and Cl^-, and 9.4×10^{-5} cm^2 s^{-1} for H^+); ΔC is the concentration gradient in pmol cm^{-3}; and Δx is the distance between the two points, measured in cm. Proton gradients were measured in solutions containing Hepes buffer and were calculated taking into account the buffering capacity of Hepes, as described in detail elsewhere (Donini and O'Donnell, 2005; Kunkel et al., 2001; Smith and Trimarchi, 2001).

Analysis of interference of pharmacological inhibitors on the functioning of ion-selective microelectrodes (ISMEs)

An ion-selective microelectrode was positioned in a dish containing 0.5 mmol l^{-1} NaCl (Na^+ and Cl^- ISMEs) or 0.5 mmol l^{-1} NaCl plus 1 mmol l^{-1} Hepes at pH 8.0 (H^+ ISME), the same solutions in which measurements of ion gradients at anal papillae were performed. The raw voltage reported by the ISME was recorded every minute for 17–20 min. At 5 min after beginning the recordings, a pharmacological inhibitor was added to the solution. After an additional 5–8 min, the solution was replaced with fresh solution (no inhibitor). The effects of inhibitors on the slope of the ISME (response of ISME to a 10-fold change in ion concentration) were assessed by comparisons between the standard calibration solutions (detailed above) and calibration solutions that contained the pharmacological inhibitors. Potential effects of the vehicle, DMSO, on the response of the ISMEs were assessed in a similar manner.

Ion-transport inhibitors

The inhibitors, their target and their final doses in the bath were as follows: ouabain – Na^+/K^+-ATPase, 1 mmol l^{-1} (Sigma-Aldrich, Oakville, Canada, cat. no. 03125); bafilomycin A1 – V-type H^+-ATPase, 1 μmol l^{-1} (LC Laboratories, Woburn, USA, cat. no. B-1080); 5-(*N*,*N*-hexamethylene)-amiloride (HMA) – Na^+/H^+ exchangers, 100 μmol l^{-1} (Sigma-Aldrich, cat. No. A9561); phenamil – Na^+ channel, 100 μmol l^{-1} (Sigma-Aldrich, cat. no. P203); bumetanide – NKCC, 10 μmol l^{-1} (Sigma Aldrich cat. no. B3023); methazolamide – carbonic anhydrase, 100 μmol l^{-1} (Sigma Aldrich cat. no. M4156); and SITS and DIDS – Cl^-/HCO_3^- antiport, 100 μmol l^{-1} (Sigma Aldrich cat. no. A0554 and D3514). The doses of the inhibitors were selected based on previous invertebrate or vertebrate tissue studies such that they were selective for their intended targets [phenamil (Reid et al., 2003; Kleyman and Cragoe, 1988); ouabain (MacVicker et al., 1993); bafilomycin (Beyenbach et al., 2000); methazolamide, DIDS and SITS (Smith et al., 2010; Nguyen and Donini, 2010); HMA (Kleyman and Cragoe, 1988; Tsui et al., 2009)]. All inhibitors were dissolved in DMSO to yield stock solutions that were subsequently diluted in bathing solution as described above.

Measurement of ion gradients adjacent to the surface of the anal papillae and effects of pharmacological ion-transport inhibitors

An *in vitro* larval anal papillae preparation was developed following the same principles outlined for measurements of ion gradients adjacent the surface of larval midge anal papillae (Nguyen and Donini, 2010). The preparation consisted of the intact four-most-posterior segments of the larva, the siphon and the four anal papillae. The open segment was sealed with Vaseline, and the preparation was held in a 35 mm Petri dish lid (Sarstedt, Quebec, Canada) containing 4 ml of a bulk bathing solution. For measurement of Na^+ and Cl^- gradients, the bathing solution was 0.5 mmol l^{-1} NaCl, and, for H^+ gradients, it was 1 mmol l^{-1} NaCl plus 1 mmol l^{-1} Hepes at pH 8.0. Previous studies using an *in vitro* anal papillae preparation of *Aedes aegypti* revealed that the magnitudes of Na^+, Cl^- and H^+ fluxes along the length of the papillae are consistent (Donini and O'Donnell, 2005). Initial measurements at four equally spaced sites

along an individual anal papilla were taken to establish the baseline magnitude ion flux (Na^+, Cl^- or H^+). This was followed by replacement of 4 µl or 40 µl of bathing solution with DMSO (resulting in exposure at 0.1% and 1% DMSO, respectively) and the preparation incubated for 15 min. At up to 1%, DMSO has no effect on the voltage reported by the ISME. Measurements were taken at the original four equally spaced sites along the papilla. At this juncture, two approaches were employed based on whether the pharmacological inhibitor and the dosage to be applied interfered with the proper function of the ISME as determined by assessing interference of the inhibitor on the ISME responses (see above).

Procedure 1: for the doses of those pharmacological inhibitors that affected the ISME response (methazolamide, SITS, DIDS), the ISME was subsequently removed from the bathing solution, and the bathing solution containing DMSO was replaced with fresh bathing solution. This was immediately followed by replacement of 4 µl or 40 µl of bathing solution with a pharmacological ion-transport inhibitor at the desired dose. The preparation was incubated for 15 min, the bathing solution replaced with fresh bathing solution (no inhibitor) and measurements immediately taken at the original four sites.

Procedure 2: for the doses of those pharmacological inhibitors that did not affect the voltage reported by the ISME (bafilomycin, ouabain, bumetanide), the bathing solution containing DMSO was replaced with fresh bathing solution and immediately followed by replacement of 4 µl or 40 µl of bathing solution with a pharmacological ion-transport inhibitor at the desired dose without removing the ISME from the bath. The preparation was incubated for 15 min, and measurements were taken at the original four sites.

Procedure 3: a third approach was necessary for assessing the effects of the amiloride derivatives HMA and phenamil because simply replacing the bath solution still resulted in ISME interference – presumably from residual HMA and phenamil. For these inhibitors, whole larvae were incubated for 15 min in 1% DMSO (control), HMA or phenamil at the desired dose in 0.5 mmol l^{-1} NaCl. Larvae were blotted dry on filter paper and subsequently set up in the *in vitro* assay. Measurements were immediately taken from the anal papillae. No interference with the ISME was seen when this procedure was utilized.

Representation and statistical analyses of data

For procedures 1 and 2 above, calculated flux values (pmol cm^{-2} s^{-1}) were analyzed by comparing baseline fluxes, the fluxes after addition of DMSO and the fluxes after addition of a pharmacological inhibitor with a one-way ANOVA ($P<0.05$). This was followed by a Bonferroni multiple comparisons test ($P<0.05$). For graphical representations of these data, the calculated flux values for DMSO and pharmacological inhibitor were expressed as a percentage of the calculated baseline flux values. Statistically significant differences between application of DMSO and pharmacological inhibitor are indicated with an asterisk at the inhibitor data bar.

For procedure 3, the calculated flux values (pmol cm^{-2} s^{-1}) of the three groups (DMSO, HMA, phenamil) were compared with a one-way ANOVA ($P<0.05$). This was followed by a Dunnett's multiple comparisons test, where the HMA and phenamil groups were compared with the DMSO control group. Statistically significant differences between DMSO and a pharmacological inhibitor are indicated with an asterisk at the inhibitor data bar.

RESULTS

The effects of pharmacological inhibitors on the response of the ISMEs were assessed before designing experiments on the anal papillae of larval mosquitoes. There was no change in the raw voltages or the slopes reported by the Na^+, Cl^- or H^+ ISMEs in the presence of bafilomycin, bumetanide or ouabain at the doses ultimately used for experiments on the anal papillae. Methazolamide, SITS and DIDS interfered with the raw voltages reported by both ISMEs based on Cl^- ionophore I and Cl^- ionophore II. The inhibitors caused the voltages to decrease (as if the ISMEs were detecting more Cl^-) and reduced the slopes by ~30–50 mV at the doses ultimately used for experiments on the anal papillae. Similar interference was seen with the amiloride derivatives HMA and phenamil on the voltages reported by the Na^+ ISME, where, in this case, voltages increased (Fig. 1) and slopes decreased. The effects of HMA and phenamil were dose dependent, in which 1 µmol l^{-1} did not appear to alter the ISME response by more than 5 mV, but 10 µmol l^{-1} altered the ISME response by ~80 mV and ~35 mV, respectively (Fig. 1).

Bafilomycin (1 µmol l^{-1}), a specific inhibitor of the V-type H^+-ATPase, decreased H^+ secretion and Na^+ absorption at the anal papillae by 55% and 41%, respectively, when compared with baseline secretion and absorption rates. DMSO had no effect on the rates of H^+ secretion or Na^+ absorption on these same papillae.

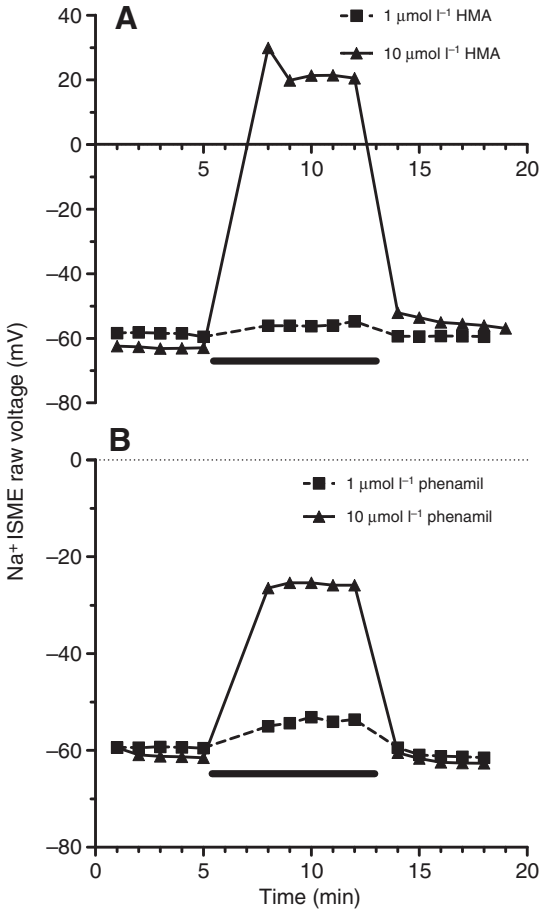

Fig. 1. Effect of HMA and phenamil on the voltage reported by the Na^+ ion-selective microelectrode (ISME). The tip of a Na^+ ISME was placed in 0.5 mmol l^{-1} NaCl solution along with the reference bridge. The voltage was recorded for 5 min and either HMA (A) or phenamil (B) was added to the solution at the indicated dose. The voltage was recorded for an additional 5–7 min in the presence of the inhibitor (black bar), and the solution was subsequently replaced with fresh 0.5 mmol l^{-1} NaCl. Voltage was recorded for an additional 5 min.

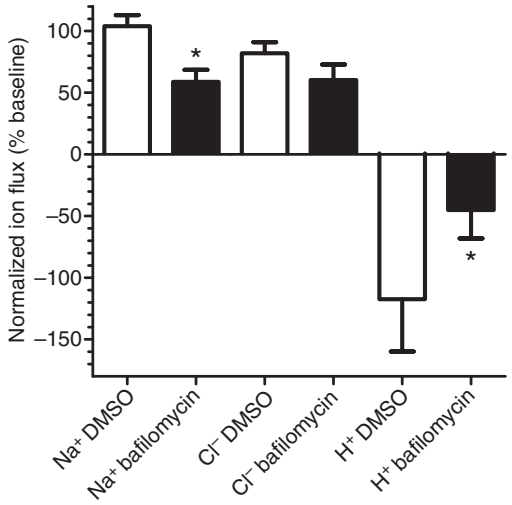

Fig. 2. Results of application of the V-type H^+-ATPase inhibitor bafilomycin ($1\,\mu mol\,l^{-1}$) on ion fluxes recorded at the anal papillae with the scanning ion-selective electrode technique (SIET). Flux values are expressed as a percentage of the baseline fluxes. Positive flux values (for Na^+ and Cl^-) indicate the influx of ions from the external bath medium into the papilla lumen, whereas negative flux values (for H^+) denote the efflux of ions from the lumen into the external bath. Data are the means ± s.e.m. of N=9–10 papillae (each from a distinct individual larva). Asterisks denote a significant difference between bafilomycin treatment and the control (DMSO). Statistical tests were conducted on the raw flux data before converting the data to a percentage of baseline fluxes (ANOVA conducted on baseline, DMSO and bafilomycin, followed by Bonferroni multiple comparisons tests, P<0.05).

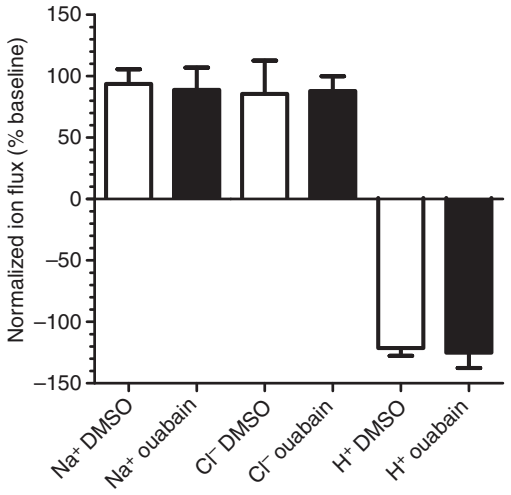

Fig. 3. Results of application of the Na^+/K^+-ATPase inhibitor ouabain ($1\,mmol\,l^{-1}$) on ion fluxes recorded at the anal papillae with the scanning ion-selective electrode technique (SIET). Flux values are expressed as a percentage of the baseline fluxes. Positive flux values (for Na^+ and Cl^-) indicate the influx of ions from the external bath medium into the papilla lumen, whereas negative flux values (for H^+) denote the efflux of ions from the lumen into the external bath. Data are the means ± s.e.m. of N=7–11 papillae (each from a distinct individual larva). Statistical tests were conducted on the raw flux data before converting the data to a percentage of baseline fluxes (ANOVA conducted on baseline, DMSO and ouabain, followed by Bonferroni multiple comparisons tests, P<0.05).

Bafilomycin apparently reduced Cl^- absorption by 40% (Bonferroni P<0.05 compared with baseline fluxes); however, DMSO reduced Cl^- absorption by 20% on the same papillae, and, as a result, there was no significant difference between the DMSO control and bafilomycin application (see Fig. 2). Neither ouabain ($1\,mmol\,l^{-1}$) nor bumetanide ($10\,\mu mol\,l^{-1}$), specific inhibitors of the Na^+/K^+-ATPase and NKCC co-transporters, respectively, altered ion absorption or secretion by the anal papillae (Figs 3 and 4).

Initial experiments employing $1\,\mu mol\,l^{-1}$ of HMA (Na^+/H^+ exchange inhibitor) and phenamil (Na^+ channel inhibitor) with procedure 2, where the inhibitor was added to the papilla preparation without removing the ISME, resulted in no changes in Na^+ absorption (N=6 for HMA, N=6 for phenamil; results not shown). Additional experiments with $10\,\mu mol\,l^{-1}$ of the inhibitors with procedure 1, where the inhibitor was added to the papilla preparation after removing the ISME from the bath, also resulted in no changes in Na^+ absorption (N=10 for HMA, N=7 for phenamil; results not shown). Application of $100\,\mu mol\,l^{-1}$ of the inhibitors with procedure 1 resulted in apparent interference on the Na^+ ISME. Therefore, whole larvae were incubated in $100\,\mu mol\,l^{-1}$ of HMA or phenamil, and Na^+ absorption rates were subsequently measured from *in vitro* preparations of these larvae. HMA had no effect on Na^+ absorption compared with DMSO controls, and phenamil decreased Na^+ absorption (Fig. 5). After incubation with phenamil, four of 11 preparations resulted in secretion of Na^+ from the anal papilla, whereas all preparations from larvae incubated in HMA resulted in Na^+ absorption at rates similar to those of the DMSO controls.

Methazolamide ($100\,\mu mol\,l^{-1}$), a carbonic anhydrase inhibitor, reduced Cl^- absorption by 79% and reduced H^+ secretion by 74% (Fig. 6). DMSO had no effect on either Cl^- absorption or H^+ secretion on the same anal papillae. The SITS and DIDS ($100\,\mu mol\,l^{-1}$) inhibitors of Cl^-/HCO_3^- exchangers reduced Cl^- absorption (Fig. 6). DMSO had no effect on Cl^- absorption on the same anal papillae treated with either SITS or DIDS. Treatment with SITS resulted in an 80% reduction in Cl^- absorption, whereas treatment with DIDS resulted in a 39% reduction of Cl^- absorption.

DISCUSSION

Overview

The anal papillae of larval mosquitoes are organs that permit survival in dilute freshwater habitats by actively absorbing NaCl and therefore limiting dilution of the hemolymph through passive loss of ions and osmotic influx of water. Studies on whole-body ion fluxes have provided insight into the ion-transport mechanisms in the anal papillae but were limited because whole-body flux measurements result from the combined actions of all the ion-transporting organs such as the Malpighian tubules and gut (Stobbart, 1965; Stobbart, 1967; Patrick et al., 2001; Patrick et al., 2002a; Patrick et al., 2002b). More recently, direct measurements of ion flux at the anal papillae were acquired through the application of a relatively novel methodology, SIET (Donini and O'Donnell, 2005; Donini et al., 2007). The present study utilized the SIET with application of pharmacological inhibitors of ion transport to provide functional evidence for specific ion-transport mechanisms responsible for the uptake of NaCl in the anal papillae of a mosquito. This study identified apical transport processes, and the results provide a basis for constructing a model of Na^+ and Cl^- transport mechanisms in the anal papillae epithelium.

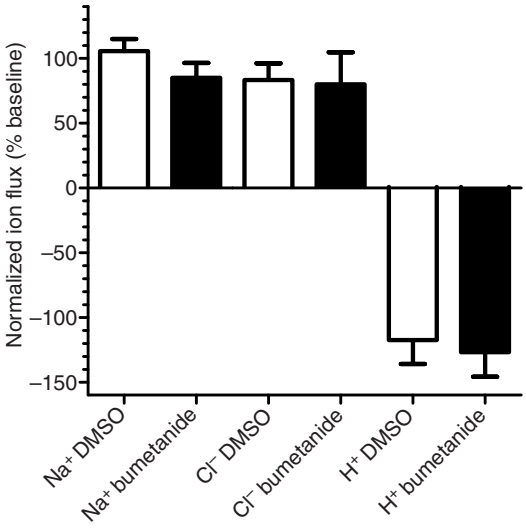

Fig. 4. Results of application of the NKCC inhibitor bumetanide (10 μmol l^{-1}) on ion fluxes recorded at the anal papillae with the scanning ion-selective electrode technique (SIET). Flux values are expressed as a percentage of the baseline fluxes. Positive flux values (for Na^+ and Cl^-) indicate the influx of ions from the external bath medium into the papilla lumen, whereas negative flux values (for H^+) denote the efflux of ions from the lumen into the external bath. Data are the means ± s.e.m. of *N*=11–23 papillae (each from a distinct individual larva). Statistical tests were conducted on the raw flux data before converting the data to a percentage of baseline fluxes (ANOVA conducted on baseline, DMSO and inhibitor, followed by Bonferroni multiple comparisons tests, $P<0.05$).

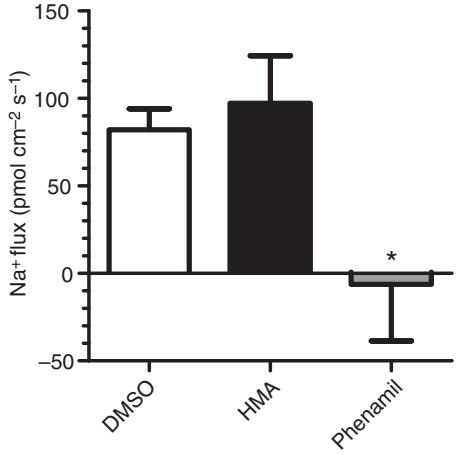

Fig. 5. Results of application of the Na^+/H^+ exchange inhibitor HMA (100 μmol l^{-1}) and the Na^+ channel inhibitor phenamil (100 μmol l^{-1}) on Na^+ fluxes recorded at the anal papillae with the scanning ion-selective electrode technique (SIET). Data are means ± s.e.m. of *N*=7–11 papillae (each from a distinct individual larva). The asterisk denotes a significant difference between inhibitor treatment and the control (DMSO). (ANOVA conducted on DMSO, HMA and phenamil groups, followed by Bonferroni multiple comparisons tests, $P<0.05$.)

Application of ion-transport inhibitors with SIET (potential for interference on ISMEs)

Before initiating experiments on the anal papillae, the potential effects of ion-transport inhibitors on the ISMEs were assessed. The results indicated that care must be taken when applying methazolamide, SITS, DIDS and amiloride derivatives in conjunction with the SIET at low ion bath concentrations. In particular, the amiloride derivatives demonstrated substantial interference with the ISME based on the Na^+ II ionophore (see Fig. 1). The effects of amiloride derivatives on Na^+ ISMEs based on other Na^+ ionophores have also been documented (Messerli et al., 2008). The effects of carbonic anhydrase and Cl^- transport inhibitors on Cl^- ISMEs have also been documented (Messerli et al., 2008). The results indicate that measurement of ion fluxes with SIET should not be attempted while methazolamide, SITS, DIDS and amiloride derivatives are present in the bath, and instead tissues should be incubated with these inhibitors, and the inhibitor should be removed from the bath just before measuring ion fluxes (see Wu et al., 2010). It is difficult to ascertain the exact effects of the inhibitors on the resulting measurements if these measurements were attempted in the presence of the inhibitors. The following potential examples are hypothesized: as the background voltage reported by the ISME will indicate that there is much more of the ion being measured in the bath than there really is (because of the inhibitor being detected by the ISME), then the resulting flux calculations could falsely indicate that there is much greater flux of the ion than there really is. By contrast, if gradients of the inhibitor develop, then the ISME will report a flux that is a combination of the ion and inhibitor gradient (a mix of both), and this will lead to either an underestimation or overestimation of ion flux. Both scenarios might be working simultaneously such that the data are even more erroneous if measurements were attempted in the presence of the drug. In addition, the slope of the ISME used in the calculation of fluxes will no longer be accurate in the presence of the inhibitor. It was determined that bafilomycin, ouabain and bumetanide did not affect ISME responses and could be directly applied to the anal papillae in the presence of the ISME.

Na^+ uptake mechanism in anal papillae

In freshwater animals, Na^+ uptake can be driven by an electrochemical gradient that is at least partially established by the V-type H^+-ATPase in the apical (water facing) membrane (Reid et al., 2003; Ehrenfeld and Klein, 1997; Yan et al., 2007). In frog skin, the gradient drives Na^+ across the apical membrane through epithelial Na^+ channels (Ehrenfeld and Klein, 1997). A similar mechanism has been shown in crustacean gill (Onken and Riestenpatt, 1998) and in fish gill (Reid et al., 2003). Another proposed mechanism for Na^+ uptake involves the Na^+/H^+ exchangers; however, it has been suggested that this mechanism would only be feasible under brackish or saline water conditions as the low Na^+ concentration in freshwater would not be sufficient for Na^+/H^+ exchange (see Parks et al., 2008). In mosquito whole-larval flux studies, Na^+ uptake was linked to H^+ secretion (Stobbart, 1971). Later, it was shown that bafilomycin inhibited Na^+ uptake by whole larvae (Patrick et al., 2002b). These studies were followed by immunolocalization of the V-type H^+-ATPase to the apical membrane of the anal papillae epithelium (Patrick et al., 2006). Finally, H^+ secretion was directly measured at the anal papillae (Donini and O'Donnell, 2005).

Together, these studies provided convincing evidence for the involvement of the V-type H^+-ATPase in Na^+ uptake. We have now shown that apically applied bafilomycin reduces H^+ secretion and Na^+ uptake that were directly measured at the anal papillae (Fig. 2), thereby confirming that V-type H^+-ATPase is, at least in part, responsible for driving Na^+ uptake by the anal papillae. Furthermore, apically applied phenamil reduced Na^+ uptake at the anal papillae,

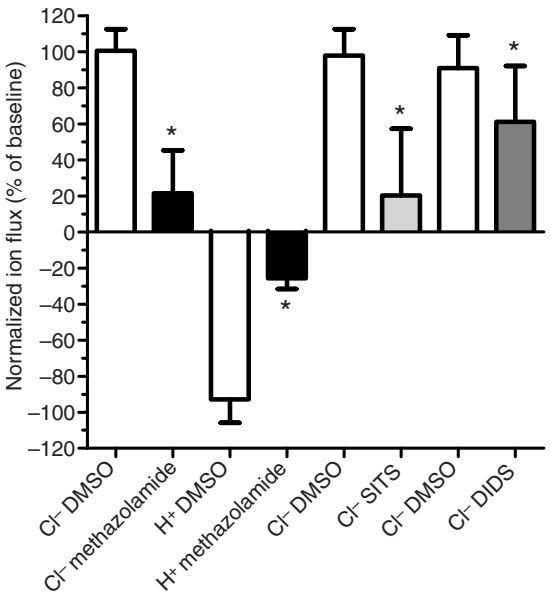

Fig. 6. Results of application of the carbonic anhydrase inhibitor methazolamide (100 μmol l^{-1}) on Cl^- and H^+ fluxes as well as Cl^-/HCO_3^- antiporter inhibitors SITS (100 μmol l^{-1}) and DIDS (100 μmol l^{-1}) on Cl^- fluxes recorded at the anal papillae with the scanning ion-selective electrode technique (SIET). Flux values are expressed as a percentage of the baseline fluxes. Positive flux values (for Cl^-) indicate the influx of ions from the external bath medium into the papilla lumen, whereas negative flux values (for H^+) denote the efflux of ions from the lumen into the external bath. Data are means ± s.e.m. of *N*=10 papillae (each from a distinct individual larva). Asterisks denote a significant difference between inhibitor treatment and the control (DMSO). The matched DMSO treatment is the bar to the immediate left of the inhibitor treatment bar. Statistical tests were conducted on the raw flux data before converting the data to a percentage of baseline fluxes (ANOVA conducted on baseline, DMSO and inhibitor, followed by Bonferroni multiple comparisons tests, $P<0.05$).

providing evidence for the presence of epithelial Na^+-channel-like transporters (Fig. 5). Together, these data suggest that the uptake of Na^+ across the apical membrane through a Na^+ channel is driven by an electrochemical gradient at least in part established by the V-type H^+-ATPase. This mechanism is apparently different from Na^+ uptake by the anal papillae of a chironomid, where the Na^+/H^+ exchanger inhibitor EIPA reduced Na^+ uptake (Nguyen and Donini, 2010). In this study, the Na^+/H^+ exchanger inhibitor HMA did not affect Na^+ uptake; however, care must be taken in excluding Na^+/H^+ exchangers because mosquitoes have been shown to express Na^+/H^+ exchangers that are not sensitive to amiloride and its derivatives (Pullikuth et al., 2006). In the integument of leeches and earthworms, Na^+ uptake has been linked to apical Na^+ channels; however, the driving force is provided by a basally located Na^+/K^+-ATPase (Weber et al., 1993; Krumm et al., 2005). The Na^+/K^+-ATPase has been localized to the basal membrane of the mosquito anal papilla epithelium (Patrick et al., 2006), and this transporter would provide a convenient means of moving Na^+ into the papilla lumen in addition to aiding the apical V-type H^+-ATPase in establishing a cytosol negative electrochemical potential that would drive Na^+ through the apical Na^+ channel. The Na^+/K^+-ATPase basal route of Na^+ transport has been established in crustacean gills (see Freire et al., 2008), the aforementioned leech and earthworm integument (Weber et al., 1993; Krumm et al., 2005), frog skin (Ehrenfeld et al., 1992) and fish gills (see Parks et al., 2008; Hwang et al., 2011). Our results could not provide functional evidence for the involvement of basal Na^+/K^+-ATPase in Na^+ uptake because ouabain had no effect on Na^+ uptake at the anal papillae (Fig. 3). This was surprising; however, two potential explanations are proposed for the result. First, all the inhibitors were applied to the apical side of the anal papillae and, hence, ouabain might not have been able to access the binding site on the basal Na^+/K^+-ATPase. Second, the anal papillae of mosquitoes might express a transporter similar to that in *Drosophila* Malpighian tubules that effectively transports ouabain, thereby protecting the Na^+/K^+-ATPase from inhibition (see Torrie et al., 2004). The structural nature of the anal papillae has thus far thwarted attempts at applying inhibitors to the basal side of the epithelium. Despite the lack of inhibition of Na^+ uptake by ouabain, it is difficult to argue against a role for the Na^+/K^+-ATPase in Na^+ uptake given its immunolocalisation to the basal membrane of the anal papillae (Patrick et al., 2006).

A third potential route for Na^+ uptake is the $Na^+/K^+/2Cl^-$ co-transporter, which has been implicated in NaCl uptake in some crustaceans (see Friere et al., 2008). A cation–chloride co-transporter has been identified at both the transcript and protein level in mosquito larvae, where expression was localized to the midgut (Filippov et al., 2003). The same study did not examine the anal papillae. Application of the cation–chloride co-transporter inhibitor bumetanide had no effect on Na^+ and Cl^- uptake measured at the anal papillae (Fig. 4). This result is in agreement with the lack of inhibition by bumetanide and furosemide on whole-larval Na^+ and Cl^- uptake (Patrick et al., 2002b). The results suggest that cation–chloride co-transport is not involved in uptake of NaCl at the mosquito anal papillae.

Cl^- uptake mechanism in anal papillae

Cl^- uptake in freshwater crustaceans occurs through Cl^-/HCO_3^- exchangers on the apical membrane, with hydration of CO_2 by carbonic anhydrase providing HCO_3^-, and apical V-type H^+-ATPase removing the resulting H^+ from the cytosol. On the basal side of the epithelium, Cl^- exits the cytosol through Cl^- channels, and this movement is supported by the combined actions of apical V-type H^+-ATPase and basal Na^+/K^+-ATPase generating a cytosol negative gradient (see Onken and Riestenpatt, 1998). For freshwater fish gills, pharmacological and molecular evidence for the involvement of Cl^-/HCO_3^- exchange in Cl^- uptake has recently been provided (Perry et al., 2009; Bayaa et al., 2009). The potential role of cation–chloride co-transporters has also been shown (Inokuchi et al., 2009; Inokuchi et al., 2008; Horng et al., 2009). In both mechanisms, the exact driving force for Cl^- uptake is still unknown. In the earthworm integument, there is pharmacological evidence for Cl^- uptake mediated by cation–chloride co-transport (Krumm et al., 2005). In the mosquito anal papillae, there is no evidence for cation–chloride co-transport involved in either Na^+ or Cl^- uptake (see above). Instead, application of the Cl^-/HCO_3^- exchanger inhibitors SITS and DIDS diminished Cl^- uptake measured at the anal papillae (Fig. 6). Furthermore, application of a carbonic anhydrase inhibitor, methazolamide, also inhibited Cl^- uptake. These results suggest that Cl^- uptake by mosquito anal papillae is mediated by Cl^-/HCO_3^- exchangers that are likely to reside on the apical membrane and that carbonic anhydrase provides HCO_3^- by hydration of CO_2. The association of Cl^- uptake with HCO_3^- secretion has also been shown with whole-larval ion-flux studies in mosquitoes (Stobbart, 1971). A similar Cl^- uptake mechanism has recently been proposed for the anal papillae of a chironomid as methazolamide reduced Cl^- uptake (Nguyen and Donini, 2010). Our results also suggest that carbonic anhydrase

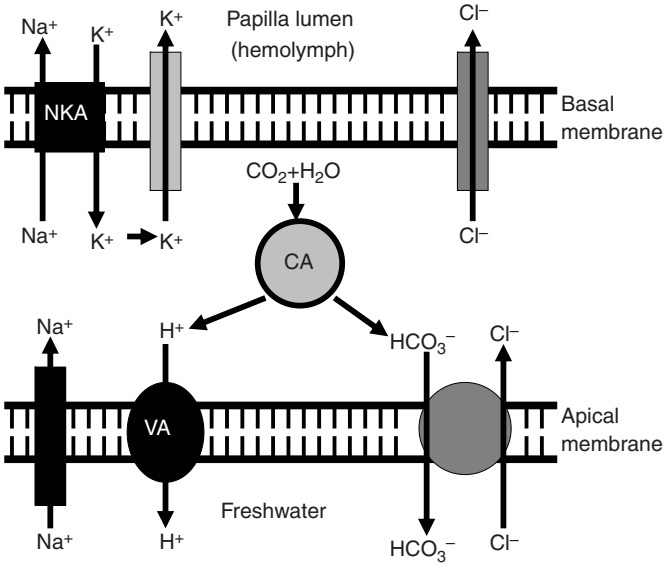

Fig. 7. Proposed model of ion-transport mechanisms responsible for NaCl uptake at the anal papillae of *Aedes aegypti*. Cytosolic carbonic anhydrase (CA) supplies H^+ to the V-type H^+-ATPase (VA) and HCO_3^- to HCO_3^-/Cl^- exchangers in the apical membrane. VA establishes an electrochemical gradient (cytoplasm negative) that drives Na^+ from the freshwater habitat into the cytoplasm of the epithelium via a Na^+ channel in the apical membrane. The Na^+/K^+-ATPase (NKA) aids the VA in producing the electrochemical gradient by transporting three Na^+ ions from the cytoplasm to the papilla lumen in exchange for two K^+ ions. The expression and location of the Na^+/K^+-ATPase was shown by Patrick and colleagues (Patrick et al., 2006). It is suggested that the electrochemical gradient (cytoplasm negative) drives Cl^- through Cl^- channels in the basal membrane from the cytoplasm into the papilla lumen (note: there is no evidence for Cl^- channels at present).

provides H^+ to the apical V-type H^+-ATPase as methazolamide reduced H^+ secretion; however, the activity of the V-type H^+-ATPase is not required for Cl^- uptake as bafilomycin had no effect on Cl^- uptake. This also suggests that Na^+ and Cl^- uptake might be independent of each other. Studies on whole-larval Cl^- uptake with bafilomycin resulted in stimulation of Cl^- uptake (Patrick et al., 2002b). This discrepancy remains unresolved; however, our measurements were taken directly at the anal papillae, whereas whole-body fluxes incorporated the combined action of all potential Cl^--uptake sites in the larvae. One can speculate whether there are additional sites of Cl^- uptake in mosquito larvae besides the anal papillae.

Summary and proposed model of NaCl uptake by anal papillae

This study has pharmacologically characterized apical Na^+ and Cl^- uptake mechanisms in the anal papillae of the larval mosquito *Aedes aegypti*. Together with previous studies on the immunolocalization of primary active transporters (Patrick et al., 2006), we propose a model for NaCl uptake mechanisms in the anal papillae (Fig. 7). Na^+ uptake occurs through apical Na^+ channels, driven by an electrochemical gradient generated by an apical V-type H^+-ATPase. Cl^- uptake occurs through apical Cl^-/HCO_3^- exchangers. Hydration of CO_2 by carbonic anhydrase provides HCO_3^- and H^+ for the anion exchanger and V-type H^+-ATPase, respectively. The Na^+/K^+-ATPase has been localized to the basal membrane (Patrick et al., 2006), and we propose that this is the likely route for Na^+ to cross the basal membrane and enter the hemolymph in the papilla lumen. It is speculated that K^+ is recycled to the hemolymph through K^+ channels in the basal membrane. Furthermore, it is possible that Cl^- crosses the basal membrane through Cl^- channels driven by the cytosol negative potential established by the basal Na^+/K^+-ATPase. This proposed mechanism of NaCl uptake by the anal papillae of *Aedes aegypti* is similar, if not identical, to that shown for numerous other freshwater animals (see above for details).

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