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The transepithelial voltage of the isolated anterior stomach of mosquito larvae (*Aedes aegypti*): pharmacological characterization of the serotonin-stimulated cells

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Summary

The lumen-negative transepithelial voltage (V_{te}) of the isolated and perfused anterior stomach of mosquito larvae (*Aedes aegypti*) was studied with a 'semi-open' preparation in which one end of the gut was ligated onto a perfusion pipette and the other end remained open to the bath. All experiments were performed with serotonin-stimulated preparations. V_{te} was abolished after addition of 2.5 mmol l^{-1} dinitrophenol and depended on the presence of Cl^-. Na^+ substitution experiments showed that a major part of V_{te} depended on the presence of this cation in the hemolymph side of the epithelium. Addition of 10 μmol l^{-1} concanamycin (78±6% inhibition) or 2.5 mmol l^{-1} ouabain (15±2% inhibition) to the bath partially inhibited V_{te}. DPC (0.5 mmol l^{-1}) or DIDS (0.1 mmol l^{-1}) reduced V_{te} when applied to the hemolymph side of the epithelium (to 49±8% or 78±3% of the control, respectively). When present on both sides of the epithelium, these inhibitors caused further V_{te} reductions (to 23±4% or 35±4% of the control, respectively). Hemolymph-side furosemide (0.1 mmol l^{-1}) or $BaCl_2$ (5 mmol l^{-1}) reduced V_{te} by 13±3% or 23±4% of the control, respectively. When applied to the hemolymph side of the epithelium, amiloride (0.2 mmol l^{-1}) significantly decreased V_{te} by 35±6% of the control, whereas the drug caused no further effect when it was subsequently also applied to the luminal side of the epithelium. The above results are the basis for an extended model for the cellular mechanisms of $NaHCO_3$ secretion/HCl absorption involved in alkalization of the anterior stomach of mosquito larvae.

Key words: *Aedes aegypti*, inhibitor, ion substitution, larva, mosquito, stomach, transepithelial voltage.

Introduction

The ion transport characteristics of the insect midgut are important for maintaining appropriate ion concentrations and pH for digestion and absorption. The luminal pH of caterpillar and larval mosquito midgut can be as high as 12 (Senior-White, 1926; Dadd, 1975; Dow, 1984). The digestive enzymes are adapted to this high pH (Eguchi et al., 1990), which is thought to dissociate tannin–protein complexes, leading to an increased ability to assimilate and utilize dietary protein (Berenbaum, 1980). The pH of the midgut is also of importance for the susceptibility to biological control agents such as *Bacillus thuringiensis* (Knowles, 1994).

Most information about insect midgut ion transport is based on studies of the midgut of lepidopteran larvae (for reviews, see Dow, 1986; Clements, 1992; Klein et al., 1996), where an electrogenic V-type H^+-pump hyperpolarizes the apical plasma membranes of the epithelial cells (for a review, see Wieczorek et al., 1999). This electrical potential is thought to energize cation secretion and luminal alkalization by an electrogenic $K^+/2H^+$ antiporter (Wieczorek et al., 1991; Azuma et al., 1995; for reviews, see Lepier et al., 1994; Wieczorek et al., 1999). However, this hypothesis has been questioned on thermodynamic grounds (Moffett and Cummings, 1994; Klein et al., 1996; Küppers and Bunse, 1996; Clark et al., 1998).

Like the lepidopteran midgut, the anterior midgut [or 'anterior stomach' in the terminology of Clements (1992), used in this and previous publications from this laboratory] of the larval mosquito is known as an alkalizing organ, and apparently the process is regulated by a neural or hormonal mechanism (Dadd, 1975, 1976). Gill et al. (1998) isolated cDNA clones of two V-ATPase subunits from the stomach of *Aedes aegypti* larvae, and a high level of expression of one of these subunits was observed in the anterior stomach (Filippova et al., 1998). Zhuang et al. (1999) showed that V-ATPases are localized on the basal membranes of the anterior stomach, whereas the ATPase is found in the apical membrane of the caeca and posterior stomach. The anterior stomach alkalization could be visualized in the living animal by allowing it to ingest pH indicators added to the rearing medium. When the rearing medium also contained inhibitors of V-ATPase, carbonic anhydrase or anion exchangers, alkalization of the anterior stomach was found to be inhibited (Zhuang et al., 1999; Boudko et al., 2001a; del Pilar Corena et al., 2002). Boudko et al. (2001b) used a semi-intact preparation of mosquito larvae

and showed that the basal acid efflux was inhibited by bafilomycin A, a specific inhibitor of V-ATPases (Dröse and Altendorf, 1997). The highest acid and chloride effluxes from the gut of semi-intact preparations were found in the anterior stomach region and were also reduced by inhibitors of carbonic anhydrase and anion exchangers when these drugs were applied to the hemolymph side of the gut (Boudko et al., 2001a). Together, these results indicate a participation of basolateral V-ATPases, carbonic anhydrase and ion exchangers in the mechanisms of alkalization. Del Pilar Corena et al. (2002), however, studied the localization of carbonic anhydrase in the mosquito intestinal system and found one isoform of this enzyme only in the caeca and in the posterior stomach.

Clark et al. (1999) perfused isolated anterior and posterior stomach segments of *Aedes aegypti* and measured the transepithelial voltage (V_{te}). Interestingly, the anterior stomach generated a lumen-negative voltage, whereas the posterior stomach displayed a lumen-positive voltage. The polarity of the voltage measured in posterior stomach segments is consistent with cation secretion, driven by an apical V-ATPase, as described for the lepidopteran midgut (see above). The lumen-negative voltage of the anterior stomach may reflect active and electrogenic H^+ absorption across the basolateral membrane and HCO_3^- secretion across the apical membrane. The luminal accumulation of HCO_3^- could be one component of luminal alkalization (up to about pH 8.3), although an additional transepithelial absorption of H^+ would be necessary to reach luminal pH values above 10 (cf. Boudko et al., 2001a). In both segments, the initially high voltages dropped significantly but could partly be re-established by the addition of submicromolar doses of serotonin (Clark et al., 1999). In a further, more detailed study of the electrophysiological characteristics of anterior stomach segments, Clark et al. (2000) showed the presence of two different cell types. In the so-called decaying cells, the basolateral membrane voltage (V_b) almost completely depolarized after mounting and showed no recovery after application of serotonin. By contrast, V_b of the so-called stable cells depolarized only slightly after mounting and hyperpolarized when serotonin was applied. These results indicated that only the stable cells generated the V_{te} in the presence of serotonin.

In the present study, ion substitution experiments and inhibitors of transporters were used in order to obtain more information about the transport mechanisms reflected in the V_{te} generated by the serotonin-responsive cells. These studies also demonstrated the usefulness of a novel semi-open preparation of the perfused stomach.

Materials and methods

Mosquitoes

Aedes aegypti L. (Vero Beach strain) eggs were provided by Dr Marc Klowden, University of Idaho, USA, from a continuously maintained colony. Eggs were hatched and larvae were maintained in a 1:1 mixture of tap water and deionized water at 26°C and on a 16 h:8 h L:D photoperiod. The water was replaced each morning, and the larvae were fed with ground Tetramin flakes (Tetrawerke, Melle, Germany). Fed fourth-instar larvae were used in all experiments.

Solutions and chemicals

The basic saline used was based on larval *Aedes* hemolymph composition (Edwards, 1982a,b) and consisted of (in mmol l^{-1}): NaCl, 42.5; KCl, 3.0; $MgCl_2$, 0.6; $CaCl_2$, 5.0; $NaHCO_3$, 5.0; succinic acid, 5.0; malic acid, 5.0; L-proline, 5.0; L-glutamine, 9.1; L-histidine, 8.7; L-arginine, 3.3; dextrose, 10.0; Hepes, 25. The pH was adjusted to 7.0 with NaOH. Na^+-free saline was prepared by substituting NaCl by *N*-methylglucamine. Instead of 5 mmol l^{-1} $NaHCO_3$, this saline contained 3 mmol l^{-1} $KHCO_3$ (no KCl). The pH was adjusted with HCl. In Cl^--free saline, gluconates (Na^+, K^+, Ca^{2+}) or sulfate (Mg^{2+}) substituted for the chlorides. The pH was adjusted with NaOH. The above components were purchased from Sigma (St Louis, MO, USA), Fisher Scientific (Pittsburgh, PA, USA) or Mallinckrodt (Hazelwood, MO, USA). Concanamycin and diphenylamine-2-carboxylic acid (DPC or *N*-phenylanthranilic acid) were from Fluka (St Louis, MO, USA). Acetazolamide, amiloride, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and furosemide were from Sigma, and $BaCl_2$ was from Mallinckrodt. The primary solvent for concanamycin, DPC and furosemide was dimethylsulfoxide (DMSO; Sigma). The final DMSO concentration of 0.1% had no effect on the V_{te} (see also Clark et al., 1999).

Perfusion pipettes

Perfusion pipettes were made from glass capillary pipettes (100 µl; VWR, West Chester, PA, USA). A pull on a vertical pipette puller (model 700B; David Kopf Instruments, Tujunga, CA, USA) was followed by manual elaboration of the pipette tips (approximately 100 µm in diameter) and by giving the pipette shaft an L-shaped form. The shaft of the pipette tips was covered with a thin layer of Sylgard 184 (Dow Corning, Midland, MI, USA).

Preparations and perfusion of anterior stomachs

After the larvae were killed by decapitation, the intestinal system was isolated and transferred to the bath of a perfusion chamber. The caeca and the posterior stomach were cut off, and the anterior stomach was mounted with its anterior end on the tip of the perfusion pipette, held by a micromanipulator (Brinkmann, Westbury, NY, USA). The preparations were tied in place with a fine human hair, and the posterior end of the stomach was left open (semi-open stomach preparation). The bath (volume 100 µl) was perfused by gravity flow with oxygenated salines at a rate of 15–30 ml h^{-1}. The perfusion pipette was connected *via* a set of three-way stopcocks to a push–pull multi-speed syringe pump (model 120; Stoelting, Wood Dale, IL, USA), allowing to change between infusion from a syringe with basic mosquito saline *via* the pipette and the gut into the bath and withdrawal from the bath through the

open, posterior end of the preparation into a second syringe. The rate of perfusion was 20–60 μl h^{-1}. According to the physical dimensions of anterior stomach preparations (Clark et al., 2000), this rate results in 3–9 luminal volume exchanges per minute. Semi-open stomach preparations were only used for further experiments if they showed a marked increase of V_{te} with serotonin (see Results; cf. Clark et al., 1999, 2000).

Electrophysiological measurements

The bath and the pipette, reflecting the interior and exterior sides of the semi-open stomach preparation, were connected *via* agar bridges (3% agar in 3 mol l^{-1} KCl) to calomel electrodes. The V_{te} was measured in the lumen with reference to the bath with the voltmeter of a voltage clamp (VCC 600; Physiological Instruments, San Diego, CA, USA) and continuously recorded on a chart recorder (model 500; Linear Instruments, Reno, NV, USA).

Electrophysiology of a semi-open tubular epithelium

The theoretical concept for the voltage generated by active and electrogenic transport across an epithelium bathed on both sides with identical salines is based on an equivalent electrical circuit where a circular current is generated by a transcellular electromotive force (E_c) and flows *via* a transcellular conductance (G_c) and a paracellular conductance (G_p). The transepithelial electrical potential difference (PD_{te}) generated by the epithelium is then determined by:

$$PD_{te} = E_c \left(\frac{G_c}{G_c + G_p} \right). \quad (1)$$

All epithelial preparations show a certain degree of damage due to preparation and mounting. In preparations of flat epithelia in Ussing-type chambers, this is known as edge damage. In the semi-open preparation of a tubular epithelium, the open end of the tube constitutes a leak conductance (G_l), parallel to the trans- and paracellular pathways. Thus, the actual measured V_{te} is then determined by:

$$V_{te} = E_c \left(\frac{G_c}{G_c + G_p + G_l} \right), \quad (2)$$

showing that the larger G_l, the lower V_{te} becomes due to a larger voltage decrement of PD_{te}. This is even more evident from the V_{te}/PD_{te} ratio:

$$\frac{V_{te}}{PD_{te}} = \frac{G_{te}}{G_{te} + G_l}, \quad (3)$$

where G_{te} is the transepithelial conductance ($=G_c+G_p$). Only at $G_l=0$ does the V_{te}/PD_{te} ratio become 1. However, the actual degree of the voltage decrement depends not only on the presence and magnitude of G_l but also on the relationship between G_{te} and G_l. A certain G_l can significantly short-circuit the PD_{te} of a low-conductance epithelium ($G_{te} \leq G_l$), whereas the same G_l may hardly affect the PD_{te} of a high-conductance epithelium ($G_{te} \gg G_l$). The above shows clearly that this technique cannot be used with every tubular epithelium. A tight, high-conductance epithelium dominated by a large G_c is certainly favorable.

Statistics

All data are presented as means ± S.E.M. In Figs 2–6, the V_{te} time courses were normalized to percentage of the control. In these cases, the absolute control values of the shown experiments are indicated in the legends. Differences between groups were tested with the paired Student's *t*-test. In those cases where controls were followed by two experimental results (e.g. V_{te} after application of a drug to the bath and V_{te} after a subsequent change to withdrawal mode), one-way analysis of variance (ANOVA) with Tukey's *post-hoc* test was performed. Significance was assumed at $P<0.05$.

Results

In the pilot phase of the present study, it was noticed that insertion of a second perfusion pipette (as used in Clark et al., 1999, 2000) into the posterior end of the preparation did not result in marked changes of the measured transepithelial voltage (V_{te}). Even cutting off small slices of the open end of the stomach had little or no effect on V_{te} (see Fig. 1). Only as the cuts approached the pipette tip did V_{te} drop with each new cut.

The open end of the stomach preparation permitted changing the flow direction in the perfusion pipette from infusion to withdrawal mode. Changes of the flow direction were often accompanied by fast voltage transients probably due to transient changes of hydrostatic pressure (see Figs 3, 6). Sometimes, V_{te} was slightly different between infusion and withdrawal modes (see Fig. 6). In these cases, the results were always read after returning to infusion mode. In some pilot experiments, it was recognized that a change to withdrawal mode at high perfusion rate (60 μl h^{-1}) resulted in a dramatic increase of V_{te} or in its irreversible breakdown. These obvious artifacts are probably due to closure of the pipette or to damage of the tissue and could be avoided by a short perfusion stop before a direction change and by using a lower perfusion rate (20 μl h^{-1}).

When the perfusion pipette was inserted into the anterior end of the anterior stomach of *Aedes aegypti*, a high, lumen-negative V_{te} of sometimes over –100 mV was detected. This high initial voltage rapidly decreased, as previously described in Clark et al. (1999, 2000). After some minutes and after fixing the stomach preparation on the pipette, V_{te} stabilized at –9±1 mV (range –1 mV to –25 mV; N=33). Addition of serotonin (0.2 μmol l^{-1}) to the bath perfusion saline resulted in a rapid increase of the voltage, which stabilized after 15–30 min at a significantly ($P<0.05$) more negative value of –27±3 mV (range –9 mV to –65 mV; N=33; see Fig. 1).

When dinitrophenol (DNP; 2.5 mmol l^{-1}), a well-known uncoupling agent of transmembrane H^+ gradients, was added to the bath, V_{te} was rapidly abolished (from –16±2 mV to –1±0.3 mV; N=6, $P<0.05$). Interestingly, the effect of DNP on

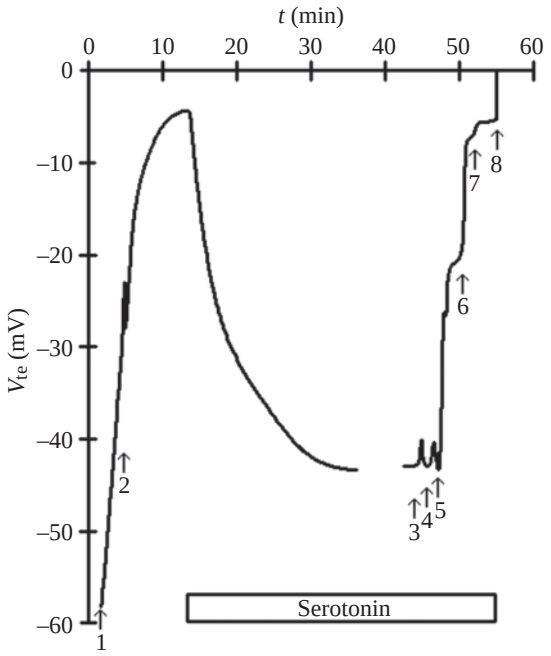

Fig. 1. Time courses of the transepithelial voltage (V_{te}) of the anterior stomach of a fourth-instar mosquito larva (*Aedes aegypti*). At arrow 1, the perfusion pipette was inserted into the anterior end of the anterior midgut. At arrow 2, the preparation was fixed on the pipette with a fine hair. At arrows 3–7, small slices (each approximately 10% of the initial length of the preparation) were cut off the open, posterior end of the preparation. At arrow 8, the remnant of the preparation was withdrawn from the pipette. During the time period indicated by the horizontal box, 0.2 μmol l^{-1} serotonin was present in the bathing medium. Infusion mode was used throughout the experiments shown.

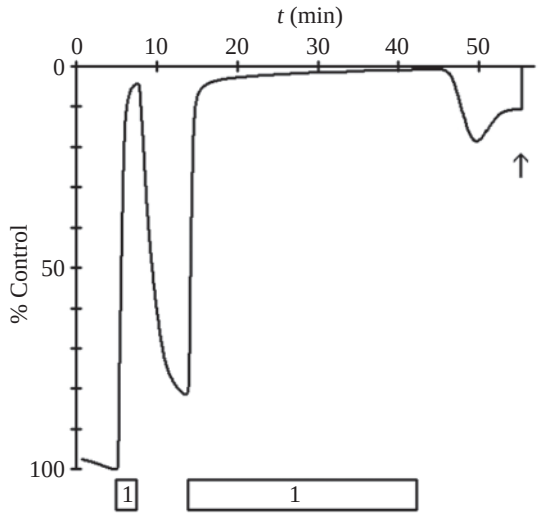

Fig. 2. Time course of the transepithelial voltage of the anterior stomach of a fourth-instar mosquito larva (*Aedes aegypti*) normalized to the percentage of the control value (–20 mV). During the time period indicated by the horizontal boxes (1), dinitrophenol (2.5 mmol l^{-1}) was present in the bath perfusate. Infusion mode was used throughout the experiment shown. At the arrow, the preparation was withdrawn from the perfusion pipette.

V_{te} was almost completely reversible if the washout was rapidly initiated (see Fig. 2). However, prolonged bath perfusion with DNP caused irreversible breakdown of V_{te}.

When Cl^--free saline was used on both sides of the epithelium (withdrawal mode) V_{te} was almost abolished (from –12±2 mV to –0.3±0.4 mV; N=6, P<0.05). In five of these experiments, Cl^- was substituted in the bath in a first step and then we changed to withdrawal mode to establish symmetrically Cl^--free solutions. In these experiments, Cl^- substitution in the bath resulted in a first significant decrease of V_{te} by 58±9%, from –13±3 mV to –6±2 mV (P<0.05), and the subsequent change to luminally Cl^--free solution almost abolished V_{te} (to 0±0.5 mV; P<0.05). The same experimental protocol as described above was also used with Na^+-free saline. A change to Na^+-free saline in the bath was followed by a major decrease of the luminally negative V_{te} to 43±12% of the control, from –27±9 mV to –8±1 mV (N=5, P<0.05). After establishing Na^+-free salines on both sides of the epithelium, a mean V_{te} of –5±1 mV (or 29±6% of the control; N=5) was measured, which is not statistically different from the voltage after Na^+ substitution in the bath only (P>0.05). Whereas Cl^- substitution consistently abolished V_{te} (inhibition by 89–100% of the control), the effect of Na^+ substitution showed a relatively large variation, inhibiting 50–92% of the control value. Examples of the effects of ion substitution experiments are demonstrated in time courses of V_{te} in Fig. 3.

Concanamycin A (10 μmol l^{-1}), a specific inhibitor of V-ATPases (Dröse and Altendorf, 1997), reduced V_{te} by 78±6%, from –23±6 mV to –6±3 mV (N=7, P<0.05), when the drug was applied to the bathing medium. The effect of the drug, varying between 46% and 94% inhibition, was not reversible (see Fig. 4). When ouabain (2.5 mmol l^{-1}), a specific inhibitor of the Na^+/K^+-ATPase (Skou, 1965), was added to the bathing medium, a minor but significant reduction of V_{te} by 15±2%, from –33±10 mV to –28±8 mV (N=8, P<0.05), was observed. The inhibition by ouabain varied between 10% and 25% and was reversible (see Fig. 5). A time course of V_{te} showing the subsequent effects of the two ATPase inhibitors is shown in Fig. 4. In this experiment, ouabain and concanamycin together almost abolish V_{te}.

Acetazolamide, an inhibitor of carbonic anhydrases (Maren, 1967), caused variable responses when added to the bathing medium (1 mmol l^{-1}). In five of the eight experiments, the drug reduced V_{te} to a variable extent (between 7% and 91% of the control value). In one case it had no effect at all, and in two other cases it even increased V_{te} to more negative values. On average, V_{te} before and after addition of acetazolamide amounted to –28±6 mV and –21±6 mV (N=8), respectively. These values are statistically not different (P>0.05).

In the next series of experiments, we used the non-specific inhibitors of anion transporters DIDS and DPC (cf. Culliford et al., 2003; Reddy and Quinton, 2002). When added to the bathing solution, DIDS (0.1 mmol l^{-1}) effected a significant

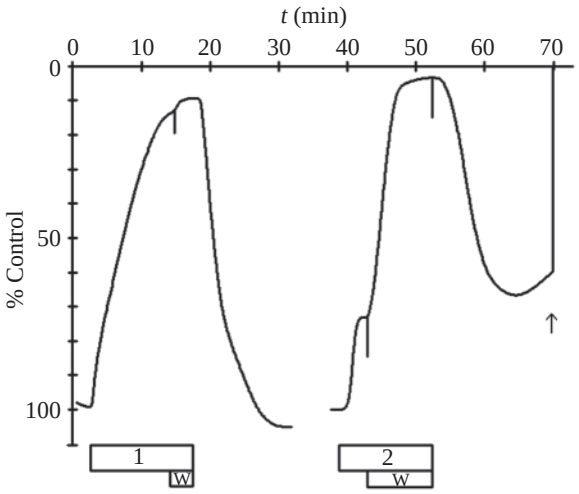

Fig. 3. Time courses of the transepithelial voltage normalized to the percentage of the control value (–38 mV and –19 mV, respectively), showing the effects of substitution of Na^+ (time period 1, *N*-methylglucamine) and Cl^- (time period 2, gluconate). During the time periods indicated by w, perfusion of the anterior stomach preparation was changed to withdrawal mode, establishing the bathing solution also on the luminal side of the epithelium. Infusion mode was used during the rest of the time. At the arrow, the preparation was withdrawn from the perfusion pipette.

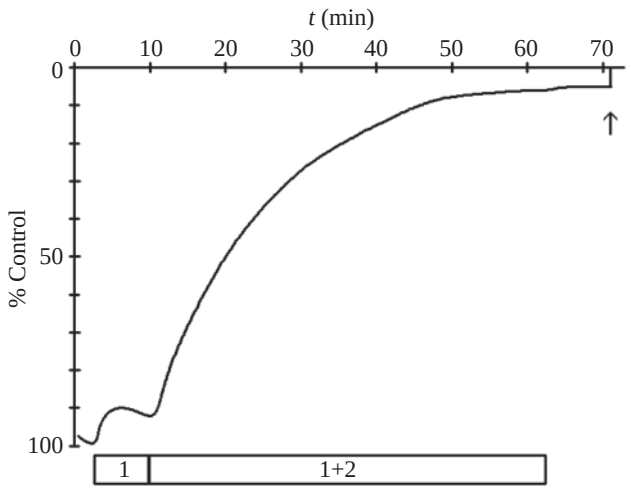

Fig. 4. Time course of the transepithelial voltage normalized to the percentage of the control value (–19 mV), showing the effects of ouabain and concanamycin A. During the time period 1, ouabain (2.5 mmol l^{-1}) was present in the bath perfusate. During the time period 1+2, ouabain (2.5 mmol l^{-1}) and concanamycin A (10 μmol l^{-1}) were present in the bath perfusate. Infusion mode was used throughout the experiments shown. At the arrow, the preparation was withdrawn from the perfusion pipette.

(*P*<0.05) decrease of V_{te} to 78±3% of the control value (from –29±10 mV to –22±7 mV; *N*=5). Changing to withdrawal mode, and thus exposing the luminal side of the epithelium to DIDS, also resulted in a further significant (*P*<0.05) decrease of V_{te} to 35±4% of the control (–10±4 mV; *N*=5). The V_{te} reduction induced by bilateral DIDS was only partially reversible. A representative time course of the effect of DIDS on V_{te} is shown in Fig. 6.

DPC (0.5 mmol l^{-1}) resulted in a decrease of V_{te} to 49±8% of the control (from –18±3 mV to –8±1 mV; *N*=11, *P*<0.05) when the drug was added to the bathing medium. The effects of the drug were, however, very variable from preparation to preparation. DPC initially caused a small, transient hyperpolarization (to 119±5% of the control value; *N*=8) of V_{te} in eight of the 11 experiments. The degree of the subsequent inhibition with respect to the control showed a very large scatter (0–80%). In five experiments, we observed the effects of DPC in the bath and after changing to withdrawal mode. In these cases, a significant (*P*<0.05) V_{te} decrease to 52±12% of the control (from –24±4 mV to –11±2 mV) after addition of DPC to the bath was followed by a second V_{te} reduction (*P*<0.05) to 23±4% of the control (–6±1 mV). The inhibitory effect of DPC was only partially reversible. An example of these experiments is shown in Fig. 6.

Barium ($BaCl_2$; 5 mmol l^{-1}), a well-known blocker of K^+ channels (Van Driessche and Zeiske, 1985), significantly reduced V_{te} by 26±5%, from –32±10 mV to –24±7 mV (*N*=6, *P*<0.05), when applied to the bathing medium. The effect of $BaCl_2$ was reversible. An example of the influence of Ba^{2+} ions is shown in a V_{te} time course together with an example of the

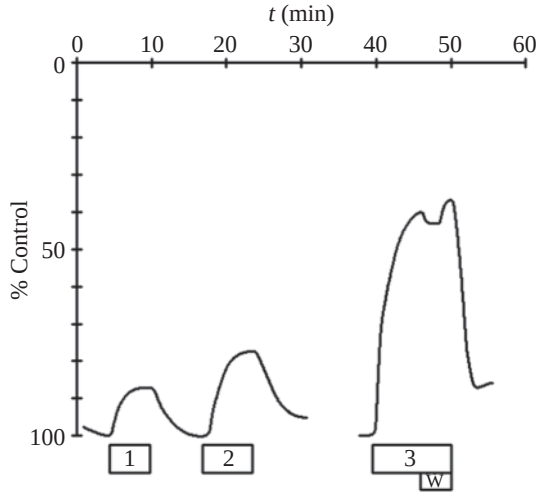

Fig. 5. Time courses of the transepithelial voltage normalized to the percentage of the control value (–46 mV and –22 mV, respectively), showing the effects of ouabain (2.5 mmol l^{-1}) during time period 1, of $BaCl_2$ (5 mmol l^{-1}) during time period 2 and of amiloride (0.2 mmol l^{-1}) during time period 3. During the time period denoted w, perfusion of the preparation was changed to withdrawal mode, establishing the bathing solution also on the luminal side of the epithelium. Infusion mode was used during the rest of the time.

effect of ouabain (see Fig. 5). A similar degree of V_{te} inhibition as with $BaCl_2$ was observed when furosemide (0.1 mmol l^{-1}), an inhibitor of $Na^+/K^+/2Cl^-$ and KCl symports as well as Cl^- channels (cf. Culliford et al., 2003), was added to the bath perfusion saline. The drug caused a significant (*P*<0.05) V_{te} decrease by 13±3%, from –23±4 mV to –20±5 mV (*N*=4).

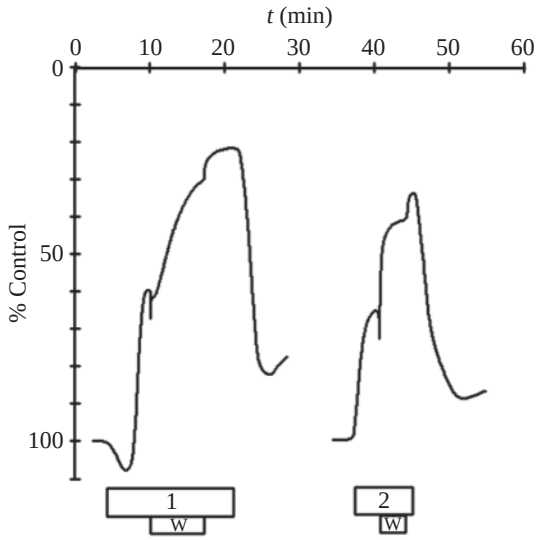

Fig. 6. Time courses of the transepithelial voltage normalized to the percentage of the control value (–29 mV and –58 mV, respectively), showing the effects of DPC (0.5 mmol l^{-1}) during time period 1 and of DIDS (0.1 mmol l^{-1}) during time period 2. During the time periods denoted w, perfusion of the preparation was changed to withdrawal mode, establishing the bathing solution also on the luminal side of the epithelium. Infusion mode was used during the rest of the time.

In 10 experiments, amiloride (0.2 mmol l^{-1}), an inhibitor of epithelial Na^+ channels and various Na^+-dependent ion exchangers (for references, see Garty and Benos, 1988) as well as of the putative insect $K^+/2H^+$ exchanger (Wieczorek et al., 1991), was applied to the bathing solution of the anterior stomach of *Aedes aegypti* larvae. The drug significantly (P<0.05) reduced V_{te} by 35±6%, from –27±6 mV to –18±5 mV (N=10). With 12–64% of inhibition, the effect of amiloride showed, however, a large variation. In five of the experiments, we changed to withdrawal mode in the presence of amiloride. After a first significant (P<0.05) V_{te} decrease by 40±10%, from –19±1 mV to –11±1 mV, with amiloride present in the bath only, the voltage stabilized at –10±2 mV in withdrawal mode with the drug on both sides of the epithelium. The voltages in the presence of amiloride in the bath and on both sides of the epithelium are statistically not different (P>0.05). An example of the effects of amiloride is shown in a V_{te} time course in Fig. 5.

Discussion

The semi-open preparation of the anterior stomach

The semi-open anterior stomach of mosquito larvae is not the first preparation of this type. A similar approach has been successfully used to analyze the transport mechanisms of NaCl absorption across the gills of hyperosmoregulating crabs (Drews and Graszynski, 1987; Onken and Graszynski, 1989). Nevertheless, it can be argued that the large scatter of the V_{te} before and after stimulation with serotonin (see Results) is related to the experimental approach. A certain dispersion of V_{te} is not uncommon and can be related to intrinsic differences between individual tissues and to the quality of individual preparations/mountings. Of course, we cannot exclude that the large scatter observed is at least partly based on methodical reasons related to the semi-open preparation. However, the mean voltages under control conditions and after stimulation with serotonin are very similar to those observed by Clark et al. (1999, 2000), who used two pipettes and, thus, a closed preparation. We observed hardly any V_{te} changes when a second pipette was inserted into the open end of the preparation or when thin slices of the open end of the gut preparation were cut off (see Materials and methods; Fig. 1). These observations show that the decrement of transepithelial electrical potential difference (PD_{te}) in the semi-open preparation is absent or at least small. The findings also indicate that the tissue is a high-conductance epithelium with a large transepithelial conductance (G_{te}). However, the high voltages generated (see Results; Clark et al., 1999, 2000) and the large ionic gradients maintained under *in vivo* conditions (Boudko et al., 2001a) show that the tissue is a tight epithelium with a low ratio between paracellular and transepithelial conductances (G_p/G_{te} ratio) and with the transcellular conductance (G_c) being much larger than G_p (cf. Schultz, 1978). Thus, the electrical characteristics of the tissue are evidently dominated by G_c.

Changes of the saline's conductance may induce V_{te} changes of a semi-open preparation without affecting the epithelium but by changing the leak conductance (G_l; cf. Materials and methods). In the present study, the conductance of the saline was certainly decreased when Na^+- or Cl^--free salines were used. However, in both cases, a decrease of V_{te} was observed that is opposite to an effect of a reduced G_l alone. Thus, the true effect on the PD_{te} could have been even larger than the V_{te} change actually observed.

The advantages of the semi-open stomach preparation are evident. The preparation itself is much easier to accomplish. Of particular importance for a very small tissue is that the technique preserves tissue by avoiding the necessity of ligating the stomach segment on a second pipette. Moreover, the open end allows manipulations on the luminal side of the epithelium using withdrawal mode, avoiding the time delay in solution changes imposed by the dead space of the perfusion system in the closed preparation.

The V_{te} *of the anterior stomach of larval* Aedes aegypti

The V_{te} generated by the isolated, anterior stomach of larval *Aedes aegypti* was measured with identical salines on both sides and it was irreversibly inhibited with dinitrophenol, an uncoupling agent that reduces mitochondrial ATP generation (Wu and Beyenbach, 2003; see Results; Fig. 2). Thus, V_{te} reflects active transepithelial ion movement. Interesting, however, is the reversibility of the effect of dinitrophenol when it was applied only for short times. Because of the capability of dinitrophenol to decrease transmembrane H^+ gradients, this observation could be interpreted as a short-circuit of a proton gradient across the basolateral membrane. Thus, this result

suggests the presence of a basolateral proton pump and its importance for the generation of V_{te} (see below).

The larval caeca and stomach receive direct serotonergic input from two large neurons located in the ingluvial ganglion (S.B.M. and D.F.M., unpublished observations). All results of the present study were obtained after stimulation with serotonin (see Results; Fig. 1). According to Clark et al. (2000), two populations of cells were encountered in the anterior stomach: (1) a cell population with a stable basolateral membrane potential (V_b) that responds to serotonin with a hyperpolarization of the basolateral and apical membranes and (2) a population of decaying cells where V_b almost completely depolarizes with time and does not recover in the presence of serotonin. Thus, these decaying cells do not contribute to the generation of V_{te} of the isolated preparation and it is evident that the results of the present study are exclusively related to the serotonin-stimulated cell population.

Which ions are transported?

The V_{te} polarity indicates transepithelial absorption of cations and/or secretion of anions. Na^+ absorption can be ruled out because hemolymph-side Na^+ substitution affected V_{te} whereas luminal substitution had no further effect (see Results; Fig. 3). Thus, V_{te} may reflect Na^+-dependent anion secretion. Substitution of internal Cl^- reduced V_{te}. However, this decrease could just reflect passive, paracellular diffusion of Cl^- from the lumen to the hemolymph side of the tissue due to the concentration gradient present under these conditions. Abolition of V_{te} was only observed when Cl^- was also substituted on the luminal side (see Results; Fig. 3). Thus, V_{te} seems not to reflect Na^+-dependent Cl^- secretion.

The observed results are, however, consistent with the assumption that V_{te} reflects HCO_3^- secretion/H^+ absorption, as has been observed with the anterior stomach region of intact larvae and semi-intact preparations, respectively (Zhuang et al., 1999; Boudko et al., 2001a,b). Like the alkalization of the anterior stomach lumen and also like the acidification of the hemolymph-facing surface of the anterior stomach, V_{te} of the isolated tissue was inhibited with a V-ATPase blocker and with non-specific inhibitors of anion transporters (see Results; Figs 4, 6; Zhuang et al., 1999; Boudko et al., 2001a,b).

Luminal alkalization and contraluminal acidification of living larvae or semi-intact preparations were observed to depend on a functioning carbonic anhydrase (Boudko et al., 2001a,b; del Pilar Corena et al., 2002), which may serve to rapidly supply H^+ and HCO_3^- to the respective transporters. Del Pilar Corena et al. (2002) localized one isoform of the carbonic anhydrase in the caeca and in the posterior stomach of *Aedes aegypti* but not in the anterior stomach. This result does not exclude the possibility that other isoforms may be present in the anterior stomach, and the above-mentioned results obtained under *in vivo* conditions clearly indicate the participation of carbonic anhydrase in stomach alkalization. However, in our experiments, even a high concentration of acetazolamide had very inconsistent effects on V_{te} (see Results). Could it be that the role of the carbonic anhydrase in alkalization of the anterior stomach is restricted to the very beginning of the anterior stomach? This could explain our findings, because this part of the stomach was used for fixing the preparation on the pipette and might have been exposed only in part of our measurements. Another possibility to explain our results with respect to acetazolamide could be related to the *in vitro* conditions used in the present study. The role of the carbonic anhydrase in alkalization of the anterior stomach of mosquito larvae and in the generation of V_{te} certainly needs to be addressed in further more-detailed studies.

Altogether, however, it seems obvious to assume that the transepithelial voltage of the isolated anterior stomach reflects HCO_3^- secretion/H^+ absorption involved in the alkalization of the stomach lumen.

Further details of the transport mechanisms

Boudko et al. (2001a) outlined a model of HCO_3^- secretion/H^+ absorption where a basolateral V-ATPase mediates transbasal H^+ absorption and generates a driving force for apical anion exchangers, resulting in transapical HCO_3^- secretion. Carbonic anhydrase rapidly supplies these transporters with their substrates. Since hemolymph-side DIDS had an effect on Cl^- efflux from the cells of the semi-intact preparation, the authors concluded that Cl^- channels might be present in the basolateral membrane. Our results are consistent with this basic outline: V_{te} depends on the presence of Cl^-, it is inhibited by hemolymph-side concanamycin, DIDS and DPC and by luminal DIDS and DPC (see Results; Figs 3, 4, 6). However, the findings of the present study add some important details, outlined in Fig. 7 and discussed below.

It is obvious that special care has to be taken when results obtained with non-specific inhibitors of anion transporters such as DIDS, DPC and furosemide are interpreted (cf. Culliford et al., 2003; Reddy and Quinton, 2002). Apart from the low specificity, these inhibitors show a significant lipid solubility and it can be argued that they not only act on the side of application but can also reach the more distant membrane. However, DIDS, DPC, furosemide and also amiloride always caused an almost immediate V_{te} decrease after their application to the bath, indicating that the site of action was at the basolateral membrane. After reaching a stabilized, reduced V_{te} in the presence of internal DIDS or DPC, these drugs caused additional V_{te} decreases when applied also to the luminal side of the epithelium, supporting the above view.

Despite the above limitations, the results of the present study are nevertheless useful to elaborate a hypothetical transport model for active and electrogenic HCO_3^- secretion/H^+ absorption that needs, of course, further confirmation by future studies. The results obtained with Na^+-free saline (see Fig. 3) indicate that a part of the transport processes depends on the presence of hemolymph-side Na^+ whereas another part does not. The Na^+-independent part could be explained by the mechanism proposed by Boudko et al. (2001a; see above). Instead of considering a so far unknown electrogenic Cl^-/HCO_3^--exchanger, we put forward the alternative idea of

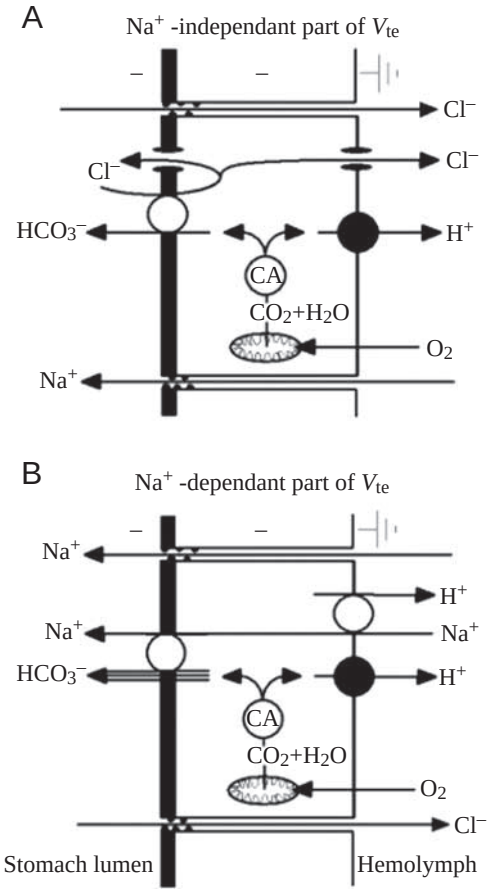

Fig. 7. Hypothetical transport model of $NaHCO_3$ secretion and HCl absorption across the serotonin-stimulated cell population in the isolated and perfused anterior stomach of mosquito larvae (*Aedes aegypti*) proposed to be reflected in the outside negative transepithelial voltage (V_{te}). (A) Na^+-independent part. Metabolic CO_2 is hydrated and dissociates, accelerated by carbonic anhydrase (CA), into H^+ and HCO_3^-. H^+ are pumped by V-ATPases across the basolateral membrane to the hemolymph, resulting in hyperpolarization of the cellular electrical potential and in high cellular HCO_3^-. Cl^-/HCO_3^- exchange across the luminal membrane is driven by the high cellular HCO_3^-. To explain the electrogenic nature of the overall process, anion channels are proposed to be present in the apical membrane, allowing Cl^- recycling and/or electrogenic secretion of HCO_3^- driven by the cellular negativity. Cl^- channels in the basolateral membrane allow transcellular absorption of Cl^- ions (cf. Boudko et al., 2001a). (B) Na^+-dependent part. In addition to energization of HCO_3^- secretion *via* apical anion exchange/channels (see A), the V-ATPase is proposed to energize transapical $NaHCO_3$ secretion *via* electrogenic $Na^+/2–3HCO_3^-$ symporters. Na^+/H^+ exchangers in the basolateral membrane are considered to supply the cells with Na^+ and to support the V-ATPases to drive H^+ across the basolateral membrane. Paracellular secretion of Na^+/absorption of Cl^- driven by V_{te} is proposed to guarantee mass transport. See Discussion for further details.

a parallel arrangement of an electroneutral anion exchanger and an anion channel (cf. Fig. 7A). This allows transapical HCO_3^- secretion *via* anion exchange that could be driven by the high cellular HCO_3^- concentration (cf. Boudko et al., 2001a) and explains the electrogenicity of the process without assuming a new type of transporter. At the almost equal concentrations of Cl^- and HCO_3^- in the tissue of anterior stomachs, even a considerable amount of HCO_3^- might be driven through the anion channels into the lumen (cf. Cuthbert, 2001). For the Na^+-dependent part of V_{te}, we favor the idea of an Na^+-dependent transapical HCO_3^- secretion *via* $Na^+/2–3HCO_3^-$ symporters (cf. Fig. 7B). Thus, hemolymph-side Na^+ substitution would affect this part of the transport by a reduction of cellular Na^+ supplied by Na^+-dependent transporters in the basolateral membrane. One of the latter transporters is most likely an Na^+/H^+ exchanger, as the V_{te} decrease with internal amiloride indicates (see Results). Apart from supplying cellular Na^+, this transporter could support the V-ATPases to extrude H^+ across the basolateral membrane. The small effects observed with furosemide (see Fig. 5) may suggest that a basolateral $Na^+/K^+/2Cl^-$ symporter also contributes to the Na^+ entry *via* the basolateral membrane. However, the effect of this drug could just reflect an effect on basolateral Cl^- channels (cf. Culliford et al., 2003). It might be argued why the Na^+-dependent part of electrogenic HCO_3^- secretion is not maintained in Cl^--free salines. However, in the presence of anion channels the cells would depolarize in Cl^--free salines and this would eliminate the driving force for the apical $Na^+/2–3HCO_3^-$ symport.

The small but fast and reversible V_{te} decreases observed with ouabain and Ba^{2+} ions (see Results; Figs 4, 5) indicate the presence of basolateral Na^+/K^+-ATPase and K^+ channels and suggest that these transporters support the V-ATPase to generate and maintain cellular negativity. A point to argue may be the relatively small effect of ouabain on a process showing such a marked Na^+ dependence. However, as long as the V-ATPase drives just as much Na^+ out of the cells (*via* the apical $Na^+/2–3HCO_3^-$ symport) as enters (*via* the basolateral Na^+-dependent transporters) the overall process is stable even without a functioning Na^+/K^+-ATPase.

The effects of certain manipulations showed a particularly high variability: whereas Cl^- substitution always almost abolished V_{te} (inhibition of 89–100%), the inhibition by Na^+ substitution (50–92%), concanamycin (46–94%), ouabain (10–25%), hemolymph-side DPC (0–82%) and amiloride (12–64%) showed much higher variations. These observations suggest that the relative proportions of Na^+-dependent and Na^+-independent HCO_3^- secretion/H^+ absorption may markedly vary between individual stomach preparations.

With mosquito saline on both sides of the epithelium, the luminally negative V_{te} could supply the driving force for paracellular movement of Na^+ from the hemolymph to the stomach lumen and/or movement of Cl^- in the opposite direction. Altogether, the serotonin-stimulated cell population could then mediate transapical HCO_3^- secretion, transbasal H^+ absorption and transepithelial Na^+ secretion and Cl^- absorption. These processes could contribute to the luminal alkalization under *in vivo* conditions. However, a 0.1 mol l^{-1} $NaHCO_3$ solution has only a pH of about 8.3, thus it is obvious

that secretion of $NaHCO_3$ alone cannot explain a luminal pH of 11. It has been repeatedly suggested (Boudko et al., 2001a,b) that an apical, amiloride-blockable $K^+/2H^+$ exchanger might be involved in stomach alkalization of mosquito larvae. In the present study, luminal amiloride did not affect V_{te} (see Results; Fig. 5). Nevertheless, it could well be that additional, transepithelial H^+ absorption by apical $K^+/2H^+$ antiports and basolateral V-ATPases is a feature of the decaying cell population. Such a process could then transform the secretion of HCO_3^- into a secretion of CO_3^{2-}, explaining the strong alkalization observed under *in vivo* conditions. It is evident that the above hypotheses need to be investigated in further more-detailed studies in the future.

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