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THE ANTERIOR AND POSTERIOR 'STOMACH' REGIONS OF LARVAL *AEDES AEGYPTI* MIDGUT: REGIONAL SPECIALIZATION OF ION TRANSPORT AND STIMULATION BY 5-HYDROXYTRYPTAMINE

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Summary

The 'stomach' region of the larval mosquito midgut is divided into histologically distinct anterior and posterior regions. Anterior stomach perfused symmetrically with saline *in vitro* had an initial transepithelial potential (TEP) of –66 mV (lumen negative) that decayed within 10–15 min to a steady-state TEP near –10 mV that was maintained for at least 1 h. Lumen-positive TEPs were never observed in the anterior stomach. The initial TEP of the perfused posterior stomach was opposite in polarity, but similar in magnitude, to that of the anterior stomach, measuring +75 mV (lumen positive). This initial TEP of the posterior stomach decayed rapidly at first, then more slowly, eventually reversing the electrical polarity of the epithelium as lumen-negative TEPs were recorded in all preparations within 70 min. Nanomolar concentrations of the biogenic amine 5-hydroxytryptamine (5-HT, serotonin) stimulated both regions, causing a negative deflection of the TEP of the anterior stomach and a positive deflection of the TEP of the posterior stomach. Phorbol 12,13-diacetate also caused a negative deflection of the TEP of the anterior stomach, but had no effect on the TEP of the posterior stomach. These data demonstrate that 5-HT stimulates region-specific ion-transport mechanisms in the stomach of *Aedes aegypti* and suggest that 5-HT coordinates the actions of the Malpighian tubules and midgut in the maintenance of an appropriate hemolymph composition *in vivo*.

Key words: *Aedes aegypti*, mosquito, larva, midgut, anterior stomach, posterior stomach, 5-hydroxytryptamine, phorbol 12,13-diacetate, transepithelial potential, ion-transport mechanism.

Introduction

The insect midgut is the site of nutrient uptake and is the first line of defense against ingested pathogens and toxins. Development of effective formulations of control agents such as *Bacillus thuringiensis* δ-endotoxins for use against medically and economically important species must take into account the midgut physiology of target insects. Studies of epithelial ion transport in many insect species, including larval mosquitoes, have neglected the midgut, despite the fact that the midgut is by far the largest epithelial organ system and is the main site of uptake of ingested ions, water and nutrients. The midgut of larval mosquitoes is divided into four regions (Clements, 1992). Within the thorax lies the cardia, followed by eight ovate globular extensions of the midgut called gastric caecae. Extending through the abdomen from the gastric caecae to the hindgut is the 'stomach', which is divided into histologically distinct anterior and posterior regions (Clements, 1992).

Most of our information about the ion-transport properties of the insect midgut come from studies on larval Lepidoptera, because studies of midgut ion transport in non-lepidopterous insects are extremely limited in scope and breadth (for reviews, see Klein et al., 1996; Dow, 1986). Diptera and Lepidoptera are members of the same superorder, the Mecopterida (Whiting et al., 1997), and larvae of these groups share the trait of a highly alkaline midgut lumen (Dow, 1986; Dadd, 1975). One might therefore expect the mechanisms of midgut ion transport to be similar in larval mosquitoes and in larval Lepidoptera. The present investigation demonstrates that the anterior stomach of *A. aegypti* is fundamentally different from the lepidopteran midgut, despite the shared trait of alkalization, and that ion transport within *A. aegypti* stomach shows a high degree of regional specialization. In addition, we demonstrate that concentrations of 5-HT previously reported in the hemolymph of *A. aegypti* larvae (Clark and Bradley, 1997) are sufficient to stimulate midgut ion transport, suggesting coordinated regulation of ion (and possibly fluid) transport in Malpighian tubules, anterior stomach and posterior stomach.

Materials and methods

Mosquitoes

Aedes aegypti eggs (Vero Beach strain) were provided by Dr Marc Klowden, University of Idaho, from a continuously maintained colony. Eggs were hatched in deionized water, and

larvae were maintained in deionized water on Tetramin flakes (Tetrawerke, Melle, Germany). Fed fourth-instar female larvae were used in all experiments.

Solutions and chemicals

The saline used in all experiments was based on a description (Edwards, 1982a,b) of the hemolymph composition of larval *A. aegypti* and consisted of the following (in mmol l^{-1}): NaCl, 42.5; KCl, 3.0; $MgSO_4$, 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. pH was adjusted to 7.0 with NaOH. Phorbol 12,13-diacetate (PE) was obtained from Research Biochemicals Inc. (Natick, MA, USA).

Perfusion pipettes

Perfusion pipettes were prepared from glass capillary pipettes (100 µl, VWR). An initial pull on a David Kopf Instruments vertical pipette puller (model 700B) was followed by hand-forging to form a pipette with a bulbous tip approximately 200 µm in diameter and 400 µm in length that could be inserted into the gut lumen and tied in place using a fine human hair.

Perfusion of gut sections

Initial experiments demonstrated that the mosquito midgut, especially the anterior stomach, is extremely sensitive to radial stretch. Attempts to perfuse the gut by applying hydrostatic pressure to one end of an isolated section therefore resulted in immediate, irreversible loss of the transepithelial potential (TEP). To address this problem, we modified a Harvard Apparatus multi-speed transmission infusion/withdrawal perfusion pump (model 902) for simultaneous infusion and withdrawal. This apparatus allowed the application of a negative pressure at one end of the isolated gut section equal to the positive pressure applied at the other end, resulting in perfusion of the gut section with a negligible distending pressure gradient across the epithelium.

Anterior and posterior regions were distinguished visually during dissection by the distinct appearance and larger diameter of the posterior stomach relative to the anterior stomach and by the presence of Malpighian tubules or gastric caecae at either end of the midgut (Fig. 1). Micromanipulators (Brinkmann) were used to hold and manipulate the infusion and withdrawal pipettes. Sections of the anterior or posterior stomach were isolated in a 1 ml bath. The withdrawal-side pipette was then inserted into the lumen and tied in place. The infusion-side pipette, connected to the voltage-sensing electrode, was inserted into the other end of the gut section and also tied, isolating a section of gut approximately 1.5 mm in length. Gut sections that showed a sudden drop in TEP as the perfusion/voltage-sensing pipette was tied, and those with obvious leaks, were rejected. The perfusion pump and bath flow were then started. The bath was perfused at a rate of approximately 15 ml h^{-1} with oxygenated saline (100 % O_2), while the lumen of the isolated gut section was perfused from anterior to posterior with identical but non-oxygenated saline at a rate of 25 µl h^{-1} or, in later experiments, 62.3 µl h^{-1}. This change in perfusion rate had no noticeable effects on TEP.

Fig. 1. (A) The aquatic larval form of *Aedes aegypti*. (B) An isolated gut of larval *A. aegypti* illustrating the anterior (ant) and posterior (post) stomach regions. The stomach illustrated (anterior + posterior) is approximately 5 mm in length.

Application of phorbol 12,13-diacetate or 5-HT

Once the initial period characterized by rapid decay of the TEP had ceased and the preparation was maintaining a relatively stable TEP, bath flow was stopped and phorbol 12,13-diacetate (PE) or 5-hydroxytryptamine (5-HT) was added to the bath and mixed. PE was applied at a final concentration of 22.3 µmol l^{-1} in 0.01 % (v/v) dimethylsulfoxide (DMSO). Controls consisted of an identical application of DMSO in saline. 5-HT was dissolved in saline.

Data acquisition and analysis

Transepithelial potentials were determined between the bath and lumen using agar bridges consisting of 4 % agar in mosquito saline, connected to Ag/AgCl electrodes. Data were acquired through a high-impedance amplifier using Sable data-acquisition software (Sable Systems, Las Vegas, NV, USA). Data were sampled at 0.1 s intervals, with each sample an average of 112 readings. Data were smoothed with a moving window filter of 11.

Results are presented as means ± S.E.M. (*N*).

Results

Electrophysiological behavior of Aedes aegypti *stomach*

Immediately following dissection, the anterior and posterior stomach were of opposite electrical polarity. A TEP of –66.4±2.78 mV (*N*=38), lumen negative, was measured when the voltage-sensing electrode was first inserted into the lumen

of the anterior stomach and tied in place. This initial TEP then declined to near −10 mV within 10–15 min, and was maintained at this reduced value for at least 50 min (Fig. 2A). Lumen-positive TEPs were never observed in a data set of more than 70 anterior stomach preparations. In contrast, the TEP of the posterior stomach was 74.9±4.65 mV (*N*=32), lumen positive, when the electrode was first inserted into the lumen (Fig. 2B). In untreated preparations, this initial TEP collapsed, rapidly at first and then more slowly, in a roughly exponential manner (Fig. 2B). Upon reaching 0 mV, the TEP continued to drift in a negative direction, and the electrical polarity of the epithelium reversed (Fig. 2B). Lumen-negative TEPs were recorded in all untreated preparations within 70 min of set-up (Fig. 2B, *N*=9). During the decay of the TEP, spontaneous electrical activity in the form of sudden negative deflections of the TEP, followed by recovery of the falling 'baseline' values, were often observed. On some occasions, these negative deflections briefly reversed the polarity of the epithelium.

For both anterior and posterior stomach, non-perfused preparations and preparations flushed with symmetrical saline before set-up showed a similar pattern consisting of a high initial TEP that decayed rapidly. Oxygenation and/or stirring of the bath did not prevent the decay of the TEP (data not shown).

Responses of the TEP to 5-HT and phorbol 12,13-diacetate

5-HT (1 μmol l^{-1}) applied to anterior stomach preparations in steady state caused a negative deflection of the TEP that required approximately 20 min to peak at a TEP that was 62.6±7.89 % of the initial value (Fig. 3; *N*=6). The TEP of the posterior stomach responded to 5-HT with a positive deflection that required 15 min to reach a peak that was 73.1±13.97 % of the initial value (Fig. 3; *N*=4). Preparations of posterior stomach that had achieved lumen-negative TEPs at the time of 5-HT application reversed polarity, returning to lumen-positive TEPs during exposure to 5-HT (Fig. 4). The TEPs of 5-HT-stimulated anterior and posterior stomach returned rapidly to pre-treatment values upon wash-out of 5-HT from the bath (data for the posterior stomach are presented in Fig. 4; data for the anterior stomach are not shown).

The effects of 5-HT on the TEP of the posterior stomach were concentration-dependent (Fig. 5). The lowest concentration tested (1 nmol l^{-1}) did not change the TEP significantly ($P>0.05$, paired Student's *t*-test of pre- and post-treatment TEP, *N*=6). Concentrations of 5-HT above 1 nmol l^{-1} caused a significant positive deflection in the TEP (Fig. 5). The response increased with increasing concentrations within the range 1 nmol l^{-1} to 0.1 μmol l^{-1} 5-HT, but no further increase in the magnitude of the response was observed as the concentration increased to 1 and 10 μmol l^{-1} (Fig. 5). There was no difference between the magnitude of the effects of 10 μmol l^{-1} 5-HT on the TEPs of preparations that had been subjected to the entire 5-HT concentration series and of 10 μmol l^{-1} 5-HT on naive preparations (concentration series, mean increase in TEP 18.3±3.10 mV, *N*=6; naive preparations, mean increase in TEP 19.8±7.64 mV, *N*=7; $P>0.85$, *t*-test). These data are therefore pooled in Fig. 5. The TEP at the time

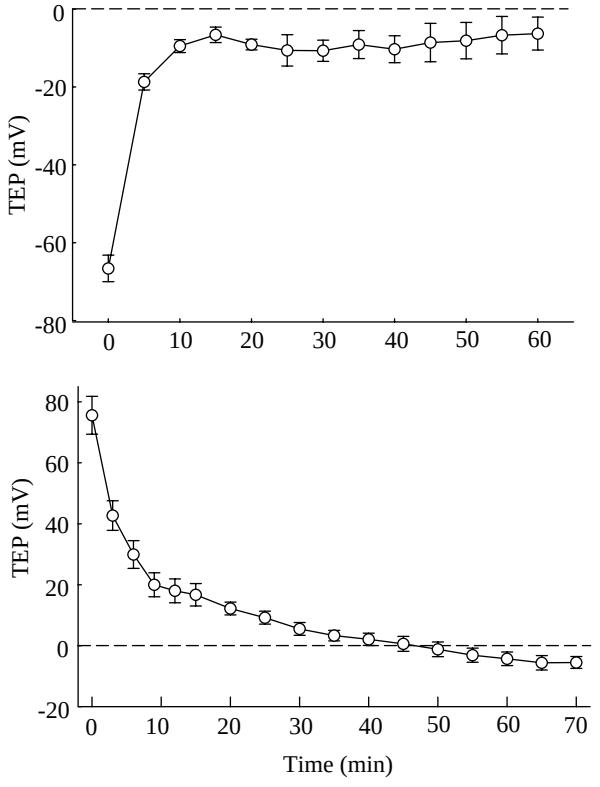

Fig. 2. (A) The transepithelial potential (TEP) of isolated, perfused anterior midgut of larval *Aedes aegypti* decays from an initial high lumen-negative value to a lower 'steady-state' value within 10–15 min. *N*=38 (0–10 min), *N*=4 (15–50 min). (B) The TEP of perfused posterior stomach of larval *A. aegypti* decays from an initial lumen-positive value to a lumen-negative value by 70 min after set-up. *N*=32 (0 min), *N*=9 (5–70 min). Values are means ± S.E.M.

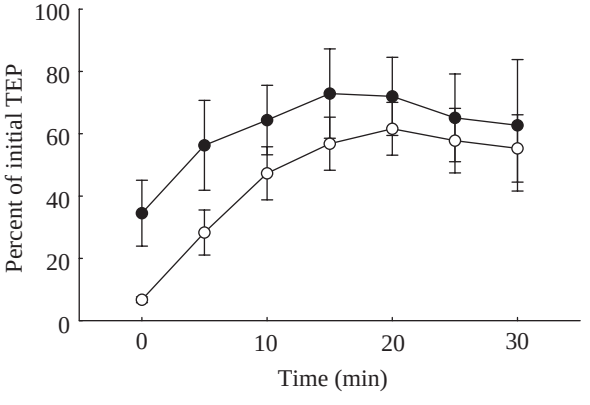

Fig. 3. 5-Hydroxytryptamine (5-HT) (1 μmol l^{-1}) partially restores the initial transepithelial potential (TEP) of both the anterior and posterior stomach. The response of each region peaks by 15–20 min following application and results in recovery to 62.6±7.89 and 73.1±13.98 % of the initial TEP, respectively, in the anterior stomach (open circles, *N*=6) and posterior stomach (filled circles, *N*=4). Values are means ± S.E.M.

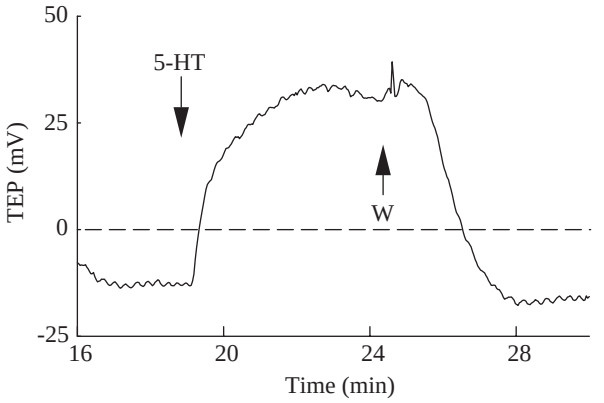

Fig. 4. 5-Hydroxytryptamine (5-HT) applied to posterior stomach preparations that had achieved a lumen-negative transepithelial potential (TEP) caused a positive deflection of the potential, reversing the electrical polarity of the epithelium; representative example of 6 experiments. 5-HT (10 nmol l^{-1}) was applied at the first arrow, and the bath flow (W) was restarted at the second arrow.

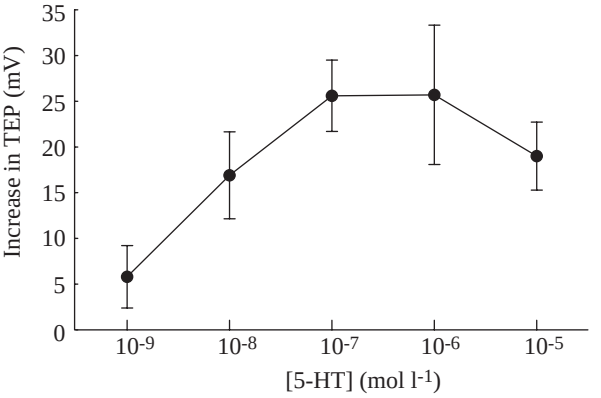

Fig. 5. Concentration-dependent effects of 5-hydroxytryptamine (5-HT) on the transepithelial potential (TEP) of perfused posterior stomach. The effects of 5-HT are presented as the difference between the TEP at the time of 5-HT application and the peak 5-HT-stimulated TEP. All concentrations except 10^{-9} mol l^{-1} caused a significant positive deflection in the TEP (P<0.05, paired t-test). N=6 for all concentrations except 10^{-5} mol l^{-1}, for which N=13. Values are means ± S.E.M.

of 5-HT application also did not appear to affect the magnitude of the response. The maximally stimulated TEPs in response to the concentration series averaged 33.0±8.61 mV, 42 % on average of the initial TEP of 82.5±10.79 mV recorded for these preparations (N=6). The anterior stomach also responded to 10 nmol l^{-1} 5-HT, although a dose–response curve was not generated for the actions of 5-HT on the anterior stomach. Sham treatments in which the bath flow was stopped for 5–10 min did not affect the TEP of untreated anterior or posterior stomach.

Phorbol 12,13-diacetate (PE, 22.3 µmol l^{-1}) caused a negative deflection in the TEP of the anterior stomach that required approximately 20 min to peak (Fig. 6). The negative deflection of the TEP persisted at a relatively constant level as long as PE was present (for at least 1 h; data not shown), and the TEP returned towards basal levels following wash-out of PE (Fig. 6). Controls consisting of 0.01 % DMSO in saline had no effect on the TEP. PE (22.3 µmol l^{-1}) had no effect on the TEP of the posterior stomach (N=4), although in several preparations it appeared to stimulate rhythmical contractile activity of the gut musculature (data not shown).

Discussion

This work describes the transepithelial potentials (TEPs) of perfused preparations of anterior and posterior stomach regions of larval *A. aegypti* midgut. Upon isolation of the anterior and posterior stomach, lumen-negative TEPs are measured in the anterior stomach region and lumen-positive TEPs are measured in the posterior stomach region. The TEPs of both the anterior and posterior stomach are initially relatively large (near 70 mV in absolute terms), but eventually decay to small lumen-negative TEPs of 5–10 mV. The decay of the initial TEPs cannot be explained by deterioration of the tissue, because the initial TEP of each region is at least partially restored upon stimulation by the biogenic amine 5-HT (serotonin). Similarly, the initial TEPs do not appear to be artifactual diffusion potentials across the epithelium since they are not affected by flushing the lumen with a symmetrical saline prior to perfusion. Instead, concentrations of 5-HT lower than those previously reported in the hemolymph of larval *A. aegypti* (approximately 10^{-7} mol l^{-1}; Clark and Bradley, 1997) are sufficient to stimulate the TEPs of the anterior and posterior stomach, suggesting that the loss of the initial TEP may be a result of the removal of the gut from stimulation by hemolymphal 5-HT *in vivo*.

The cellular mechanisms of ion transport, and their regulatory mechanisms, clearly differ in the anterior and

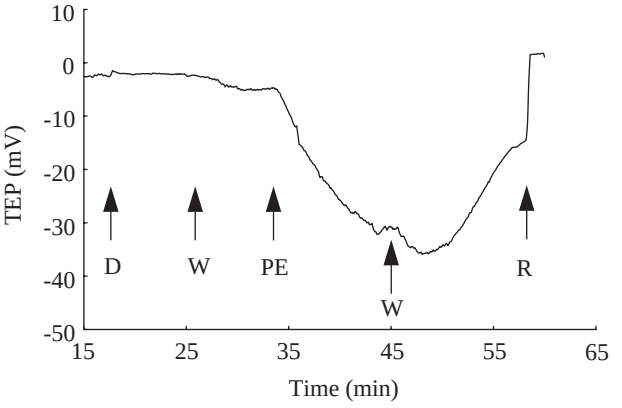

Fig. 6. Phorbol 12,13-diacetate (PE) stimulated the transepithelial potential (TEP) of isolated, perfused anterior midgut; representative example of 7 experiments. D, application of dimethylsulfoxide control; W, wash-out; PE, application of phorbol 12,13-diacetate (22.3 µmol l^{-1}); W, washout. The electrode was removed from the lumen at R.

posterior stomach of larval *A. aegypti*. Not only are the initial TEPs of opposite electrical polarity in the anterior and posterior stomach, but 5-HT causes a lumen-negative shift in the TEP of the anterior stomach and a lumen-positive shift in the TEP of the posterior stomach. Furthermore, phorbol 12,13-diacetate (PE), a protein kinase C (PKC) agonist (Castagna et al., 1982), stimulates the TEP of the anterior stomach. The PKC component of the phosphatidylinositol pathway thus appears to be involved in the regulation of ion transport by the anterior stomach and may mediate at least part of the 5-HT response. In contrast, PE does not affect the TEP of the posterior stomach, suggesting that the actions of 5-HT on the posterior stomach are independent of PKC activation.

Few studies have addressed the regulation of insect midgut ion transport by hormonal or neural factors, in contrast to the extensive literature on the regulation of other insect epithelia. Given the large size of the insect midgut relative to the rest of the animal and to the extracellular space, regulation of midgut ion transport appears to be very important in the physiology of larval *A. aegypti* and may be far more important generally in insect physiology than has been assumed. The insect midgut is highly innervated and contains, in addition to peptidergic and aminergic neurons, gastroendocrine cells containing many putative hormones. With the exception of their effects on the gut musculature, very little is known about the biological roles of these hormones (Nässel, 1988; Sehnal and Zitnan, 1996). The present study is the first to describe the stimulation of midgut ion transport by a known agonist. Dadd (1976) first suggested the possibility of regulation of midgut ion transport, describing the collapse *in vivo* of the alkaline conditions within the gut of mosquito larvae fed pH indicators upon handling, chilling or narcotization. Similarly, Clark et al. (1998) have demonstrated the collapse of alkalization upon isolation of *Manduca sexta* midgut and present evidence for the regulation of alkalization by an unknown endocrine or paracrine factor released from the basal aspect of the gut. Midgut ion transport thus appears to be regulated in both larval Diptera (present study) and Lepidoptera (Clark et al., 1998), and it is probable that midgut ion transport is regulated in other insects as well. The midgut of *Leucophaea maderae* appears to possess different functional states because it maintains a stable TEP *in vitro* that may be either lumen-positive or lumen-negative. This phenomenon is different from the short-term regulation of *A. aegypti* midgut described in the present study, because the potentials of *L. maderae* midgut do not decay but remain constant (Sacchi and Giordana, 1979).

The concentration of 5-HT found to saturate the 5-HT response of posterior midgut in the present study (10^{-7} mol l^{-1}) is the same as that previously reported in hemolymph of larval *A. aegypti* (Clark and Bradley, 1997) and is sufficient to stimulate Malpighian tubule fluid and ion secretion rates (Clark and Bradley, 1996). It seems unlikely that a regulated mechanism would operate under normal conditions at its maximal rate, rather than at a rate near the midpoint of the concentration–response curve, since this would leave no scope for increases in rate. It is also intriguing that the 5-HT concentration–response curve of Malpighian tubules (Clark and Bradley, 1996) begins at the same low concentration yet is saturated at a 10-fold greater 5-HT concentration than is that of the posterior stomach. It should be noted, however, that the larvae used by Clark and Bradley (1996, 1997) were raised in 2 % sea water, while those used in the present study were raised in distilled water, and hemolymphal 5-HT concentrations increase in response to increases in ambient salinity (Clark and Bradley, 1997). In addition, the larvae used by Clark and Bradley (1996, 1997) and in the present study were of different strains. Whatever the causes of the differences in concentration-responsiveness of midgut and Malpighian tubules in these studies, the activation of midgut ion transport by concentrations of 5-HT known to circulate in the hemolymph (Clark and Bradley, 1997) and to stimulate Malpighian tubule ion and fluid secretion rates (Clark and Bradley, 1996, 1997) suggests that the actions of the midgut and the Malpighian tubules are coordinated by hemolymphal 5-HT *in vivo*. While the exact biological role of this coordination is unknown, it presumably involves regulation of some aspect of hemolymph composition. Since hemolymphal 5-HT concentrations increase with ambient salinity (Clark and Bradley, 1997), the integration of ion transport by the midgut and Malpighian tubules to regulate hemolymphal ionic composition is an obvious possibility.

The anterior midgut of larval nematoceran Diptera (including mosquitoes) and larval Lepidoptera is characterized by a high luminal pH (Dadd, 1975; Dow, 1986). An analysis of the phylogenetic occurrence of larval midgut alkalization suggests that this trait may have arisen in the common ancestor of the mosquitoes and the Lepidoptera (T. M. Clark, unpublished observations). In any case, the shared trait of midgut alkalization and the phylogenetic closeness of mosquitoes and Lepidoptera suggest that ion transport in the anterior stomach of the mosquito may resemble that in the intensively studied midgut of larval Lepidoptera (for reviews, see Klein et al., 1996; Dow, 1986). This is clearly not the case for the primary motive force of these epithelia, as the lumen-negative TEP of *A. aegypti* anterior stomach must be generated by fundamentally different mechanisms from those that generate the lumen-positive TEP of the lepidopteran midgut.

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References

Castagna, M., Takai, Y., Kaibuchi, K., Sano, K., Kikkawa, U. and Nishizuka, N. (1982). Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* **257**, 7847–7851.

Clark, T. M. and Bradley, T. J. (1996). Stimulation of Malpighian tubules from larval *Aedes aegypti* by secretagogues. *J. Insect Physiol.* **42**, 593–602.

Clark, T. M. and Bradley, T. J. (1997). Malpighian tubules of larval *Aedes aegypti* are hormonally stimulated by 5-hydroxytryptamine in response to increased salinity. *Arch. Insect Biochem. Physiol.* **34**, 123–141.

Clark, T. M., Koch, A. R. and Moffett, D. F. (1998). Alkalinization by *Manduca sexta* anterior midgut *in vitro:* requirements and characteristics. *Comp. Biochem. Physiol.* **121**, 181–187.

Clements, A. N. (1992). *The Biology of Mosquitoes*, vol. I, *Development, Nutrition and Reproduction.* London: Chapman & Hall.

Dadd, R. H. (1975). Alkalinity within the midgut of mosquito larvae with alkaline-active digestive enzymes. *J. Insect Physiol.* **21**, 1847–1853.

Dadd, R. H. (1976). Loss of midgut alkalinity in chilled or narcotized mosquito larvae. *Ann. Ent. Soc. Am.* **69**, 248–254.

Dow, J. A. T. (1986). Insect midgut function. *Adv. Insect Physiol.* **19**, 187–328.

Edwards, H. A. (1982a). Ion concentration and activity in the haemolymph of *Aedes aegypti* larvae. *J exp. Biol.* **101**, 143–151.

Edwards, H. A. (1982b). Free amino acids as regulators of osmotic pressure in aquatic insect larvae. *J. exp. Biol.* **101**, 153–160.

Klein, U., Koch, A. and Moffett, D. F. (1996). Ion transport in Lepidoptera. In *Biology of the Insect Midgut* (ed. M. J. Lehane and P. F. Billingsley), pp. 236–264. London: Chapman & Hall.

Nässel, D. R. (1988). Serotonin and serotonin-immunoreactive neurons in the nervous system of insects. *Prog. Neurobiol.* **30**, 1–85.

Sacchi, V. F. and Giordana, B. (1979). Ion contribution to the transepithelial electrical potential difference across the isolated midgut of the cockroach *Leucophaea maderae. Comp. Biochem. Physiol.* **64**A, 419–425.

Sehnal, E and Zitnan, D. (1996). Midgut endocrine cells. In *Biology of the Insect Midgut* (ed. M. J. Lehane and P. F. Billingsley), pp. 55–85. London: Chapman & Hall.

Whiting, M. F., Carpenter, J. C., Wheeler, Q. D. and Wheeler, W. C. (1997). The Strepsiptera problem: phylogeny of the holometabolous insect orders inferred from 18S and 28S ribosomal DNA sequences and morphology. *Syst. Biol.* **46**, 1–68.