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# The anterior stomach of larval mosquitoes (*Aedes aegypti*): effects of neuropeptides on transepithelial ion transport and muscular motility

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**Summary**

**The present investigation studied the influence of a number of neuropeptides on semi-open preparations of the isolated and perfused anterior stomach of larval *Aedes aegypti*. Effects of peptides were observed on the lumen negative transepithelial voltage ($V_{te}$) that is present with serotonin in the bath; this voltage most likely reflects active $HCO_3^-$ secretion involved in alkalization of the larval anterior stomach. The five different *A. aegypti* allatostatins (allatostatin A 1–5) all affected $V_{te}$ in almost identical ways, causing a 10–15% reduction of the voltage at $10^{-7}$ mol $l^{-1}$. *A. aegypti* neuropeptide F and proctolin reduced $V_{te}$ at submicromolar concentrations. At $10^{-6}$ mol $l^{-1}$, neuropeptide F reduced $V_{te}$ by 30% and proctolin reduced $V_{te}$ by 50%. In contrast, *A. aegypti* allatotropin, *A. aegypti* head peptides I and III and *A. aegypti* short neuropeptide F were without effect on $V_{te}$. During the investigation it was observed that the peristaltic contractions of the preparations caused a dynamic component of $V_{te}$. Peristaltic contractions and the correlated voltage fluctuations depended on the presence of serotonin. Peristaltic activity and $V_{te}$ deflections were progressively inhibited by *A. aegypti* head peptides I and III by *A. aegypti* short neuropeptide F and by *A. aegypti* neuropeptide F when the peptide concentrations were increased from $10^{-8}$ to $10^{-6}$ mol $l^{-1}$. These observations show that physiological concentrations of some of the tested neuropeptides affect two processes that require coordination: ion transport and motility of the larval anterior stomach.**

Key words: *Aedes aegypti*, allatostatin, allatotropin, anterior stomach, head peptide, larva, midgut, mosquito, neuropeptide F, peristalsis, proctolin, transepithelial voltage.

## Introduction

In addition to its conventional roles in food digestion and nutrient absorption, the stomach of mosquito larvae is of critical importance for ionic, volume and acid–base homeostasis. The longitudinal pH profile of the stomach lumen indicates a special challenge for the control of acid–base balance. The anterior stomach actively secretes alkali (and/or absorbs acid) equivalents, generating a compartment of extremely high pH (Senior-White, 1926; Dadd, 1975), which is of importance for digestion (cf. Eguchi et al., 1990; Berenbaum, 1980), but also for the susceptibility to biological control agents such as *Bacillus thuringienis* (Knowles, 1994). In the posterior stomach the pH returns to almost neutral values by recovery of alkali equivalents (and/or acid secretion).

The rates and mechanisms of acid–base relevant transport processes in the stomach of mosquito larvae are poorly understood. Alkalization in the anterior stomach seems to be reflected in a luminally negative transepithelial voltage (Clark et al., 1999, 2000) and is partly based on transapical $HCO_3^-$ secretion and transbasolateral $H^+$ absorption energized by V-type $H^+$ pumps in the basolateral membrane (Zhuang et al., 1999; Boudko et al., 2001a,b; Onken et al., 2004). Since active accumulation of luminal $HCO_3^-$ only explains alkalization up to about pH 8.5, additional transepithelial absorption of acid equivalents has to be evoked to account for alkalization to values above pH 10.

Regulatory systems involved in controlling the activities of the intestinal tract in response to feeding, and in integrating its function and whole-animal homeostasis with behavioral responses such as eating, drinking and breathing, are known only in a general way (cf. Sehnal and Zitnan, 1996). Possible regulatory inputs may come *via* innervation from the central nervous system, *via* neuroendocrine factors emanating from the central nervous system or from the numerous enteroendocrine cells, each expressing one or more peptide hormones (Brown et al., 1985). Previous studies showed that the transepithelial voltage of the isolated anterior stomach of the larval yellow fever mosquito *Aedes aegypti* declined precipitously within minutes after mounting, but could be partly restored by submicromolar concentrations of serotonin (Clark et al., 1999). In a later, more detailed study (Clark et al., 2000) two cell types were discovered. In one cell type the basolateral membrane voltage was stable after mounting of the

isolated tissue, whereas it dramatically depolarized in the other cell type. It could be that the two cell types may be related to the two processes that seem to be involved in anterior stomach alkalization (see above). With respect to regulation, the above-described results suggest that endogenous serotonin and some additional chemical messengers were critical for authentic stomach function and were lost when the stomach was isolated and perfused with artificial saline.

The present study evaluated several candidate neuropeptides for their potential roles in controlling stomach function. Initially, these studies focused on ion transport of the anterior stomach, using changes in the transepithelial voltage as an indicator of effects on ion transport. However, the muscular motility of the preparations produced a dynamic component of the transepithelial voltage, which also allowed us to evaluate myotropic effects of the peptides.

## Materials and methods

### *Mosquitoes*

*Aedes aegypti* (Vero Beach strain) eggs were provided by Dr Marc Klowden (University of Idaho, Moscow, USA) from a continuously maintained colony. Eggs were hatched and larvae were maintained in a 1:1 mixture of tapwater and deionized water at 26°C and subjected to 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. The above components were purchased from Sigma (St Louis, MO, USA), Fisher Scientific (Pittsburgh, PA, USA) or Mallinckrodt (Hazelwood, MO, USA). Serotonin and proctolin were purchased from Sigma. *A. aegypti* head peptide I (Aedae-HP-I), *A. aegypti* head peptide III (Aedae-HP-III), *A. aegypti* short neuropeptide F (Aedae-sNPF also known as Aedae-LRLFa or new head peptide) and *A. aegypti* neuropeptide F (Aedae-NPF) were provided by Dr M. R. Brown (University of Georgia, Athens, USA). *A. aegypti* allatotropin (Aedae-AT) was provided by Dr J. A. Veenstra (Université de Bordeaux, Talence, France) and five different *A. aegypti* allatostatins type A (Aedae-AST-A 1–5) were provided by Dr F. G. Noriega (University of Arizona, Tucson, USA). The amino acid sequences of the peptides used are shown in Table 1. The peptides and concentrated stock solutions in water or saline were stored at –80°C before their use in the experiments.

### *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 Knopf Instruments, Tujunga, CA, USA) was followed by manual elaboration of the pipette tips (approximately 100 μm in diameter) to give the pipette shaft an L-shaped form. The shaft of the pipette tips was covered with a thin layer of cured Sylgard 184 (Dow Corning, Midland, MI, USA) to improve the electrical seal between the pipette and the tissue.

### *Preparations and perfusion of anterior stomachs*

The larvae were decapitated and the intestinal system then 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 anterior stomach was left open (semi-open preparation; see Fig. 1). 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 3-way stopcocks to a push–pull multi-

Table 1. *Neuropeptides used in the present investigation*

| Name | Abbreviation | Sequence |
|---|---|---|
| Allatostatin A 1[1] | Aedae-AST-A 1 | SPKYNFGLa |
| 2[1] | 2 | LPHYNFGLa |
| 3[1] | 3 | RVYDFGLa |
| 4[1] | 4 | ASAYRYHFGLa |
| 5[1] | 5 | LPNRYNFGLa |
| Allatotropin[2] | Aedae-AT | APFRNSEMMTARGFa |
| Head peptide I[3] | Aedae-HP-I | QRhPPSLKTRFa |
| Head peptide III[4] | Aedae-HP-III | QRPPSLKTRFa |
| Short neuropeptide F[5] | Aedae-sNPF | APQLRLRFa |
| Neuropeptide F[6] | Aedae-NPF | SFTDARPQDDPTSVAEAIRLLQELETKHAQHARPRFa |
| Proctolin | | RYLPT |

[1]Veenstra et al., 1997; [2]Veenstra and Costes, 1999; [3]Matsumoto et al., 1989; [4]Veenstra, 1999; [5]cf. Riehle et al., 2002; [6]Stanek et al., 2002.

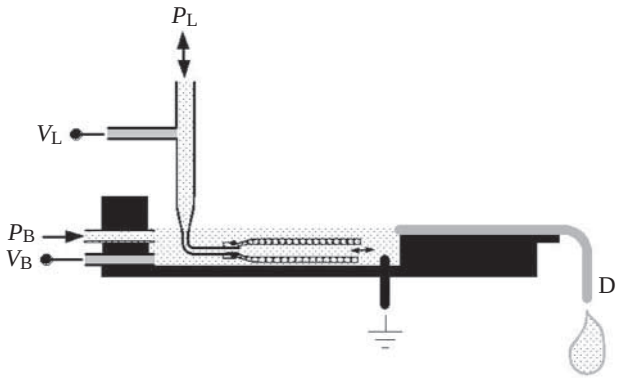


Fig. 1. Schematic representation of the set-up for the measurement of the transepithelial voltage with a semi-open preparation of stomach segments of larval mosquitoes. The luminal perfusion ($P_L$) is achieved by connection of the perfusion pipette to a push-pull multi-speed syringe pump. The direction of the perfusion can be changed by connection to the infusion syringe or the withdrawal syringe. Bath perfusion ($P_B$) is by gravity flow from interchangeable reservoirs with oxygenated salines. The transepithelial voltage is measured with calomel electrodes connected to the pipette ($V_L$) and the bath ($V_B$) by agar bridges. D, drainage.

speed syringe pump (model 120, Stoelting, Wood Dale, IL, USA). The rate of perfusion was 20–60 μl $min^{-1}$. According to the physical dimensions of anterior stomach preparations (Clark et al., 2000), this rate results in 3–9 luminal volume exchanges per minute.

### *Electrophysiological measurements*

The bath and the pipette, reflecting the hemolymph side and the lumen of the semi-open stomach preparation, were connected *via* agar bridges (3% agar in 3 mol $l^{-1}$ KCl) to calomel electrodes (see Fig. 1). The transepithelial voltage ($V_{te}$) was measured in the lumen with reference to the bath (hemolymph side of the tissue) with the voltmeter of a voltage clamp (VCC 600, Physiologic Instruments, San Diego, CA, USA) and continuously recorded on a chart recorder (model 500, Linear Instruments, Reno, NV, USA). In a prior study (Onken et al., 2004), the validity of the semi-open preparation for voltage measurements was verified and discussed in detail.

### *Statistics*

All data are presented as means ± standard error of the mean (S.E.M.). Differences between groups were tested, using one-way analysis of variance (ANOVA) with Tukey's post-test. Significance was assumed at $P<0.05$.

## Results

All experiments were started by bathing and perfusing the anterior stomach of *A. aegypti* larvae with basic mosquito saline. As described by Clark et al. (1999), we observed the initially high, luminally negative transepithelial voltage ($V_{te}$) to rapidly decrease after mounting of the tissue, suggesting washout of stimulative factors. After 5–10 min the voltage stabilized at –11±1 mV ($N$=44). In the next step, 0.2 μmol $l^{-1}$ serotonin was added to the bathing solution and only those preparations that showed a rapid and marked stimulation of $V_{te}$ under the influence of this drug were accepted for further experiments (see Fig. 2). In the presence of serotonin, the transepithelial voltage stabilized at a mean value of –35±3 mV ($N$=44).

### *Effects of neuropeptides on the transepithelial voltage in presence of serotonin*

In a first series of experiments ($N$=5), *A. aegypti* allatostatins A1 to A5 (Aedae-AST-A, see Table 1; Veenstra et al., 1997) were applied at a concentration of $10^{-7}$ mol $l^{-1}$ to the bath. All five different Aedae-AST-A affected $V_{te}$ in a very similar way, inducing a small but significant ($P<0.05$) depolarization of the luminally negative voltage by 10–15%. The voltage decreases were partly reversible. A time-course of one representative experiment is shown in Fig. 2.

In the next series of experiments, individual anterior stomach preparations were exposed to *A. aegypti* allatotropin (Aedae-AT, see Table 1; Veenstra and Costes, 1999), to two different *A. aegypti* head peptides (Aedae-HP-I, Aedae-HP-III, see Table 1; cf. Matsumoto et al., 1989; Veenstra, 1999) or to short neuropeptide F (Aedae-sNPF, see Table 1; cf. Riehle et al., 2002) at stepwise increasing concentrations between $10^{-16}$ and $10^{-6}$ mol $l^{-1}$. The time of exposure to each concentration of a peptide was between 5 and 10 min. Neither Aedae-AT ($N$=5) nor either one of the head peptides ($N$=8 and 6, respectively) nor Aedae-sNPF ($N$=5) caused a significant change of $V_{te}$ at any concentration ($P>0.05$). The results are summarized in Fig. 3.

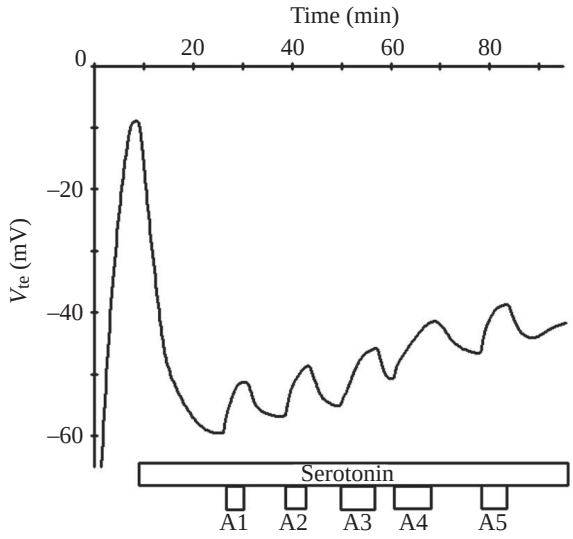


Fig. 2. Representative time-course of the transepithelial voltage ($V_{te}$) across the anterior stomach of larval *A. aegypti*, showing the initial voltage decrease after mounting the anterior stomach, the voltage stimulation induced by serotonin (0.2 μmol $l^{-1}$), and the effects of Aedae-AST-A 1–5 (example of $N$=5).

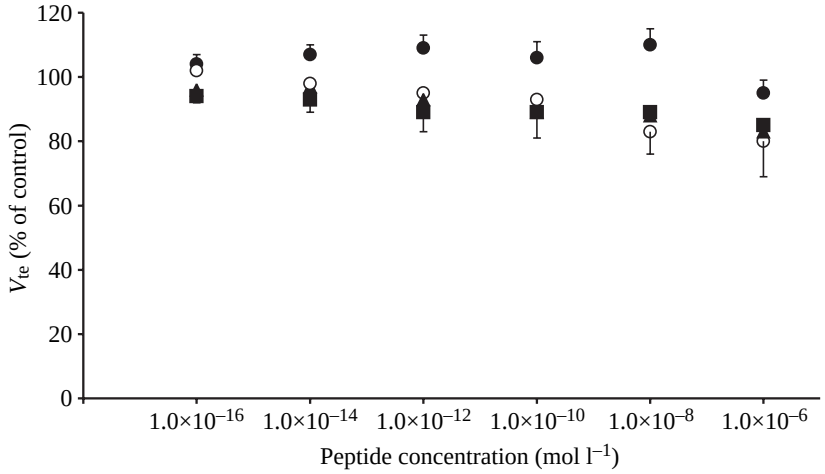


Fig. 3. Transepithelial voltages ($V_{te}$) expressed as percentage of the control voltage (100%) for different concentrations of Aedae-AT (open circles; control voltage –27±4 mV, $N$=5), Aedae-HP-I (filled circles; control voltage –27±4 mV, $N$=8), Aedae-HP-III (filled squares; control voltage –37±10 mV, $N$=6), and Aedae-sNPF (filled triangles; control voltage –43±11 mV, $N$=5). All values are means. Selected error bars in the figure reflect S.E.M.

In another series of experiments the individual tissues were exposed to increasing concentrations ($10^{-16}$ to $10^{-6}$ mol $l^{-1}$) of *A. aegypti* neuropeptide F (Aedae-NPF; see Table 1; Stanek et al., 2002). The results are summarized in Fig. 4. At $10^{-10}$, $10^{-8}$ and $10^{-6}$ mol $l^{-1}$, the peptide significantly reduced $V_{te}$ by 22±5, 26±6 and 31±7% of the control value, respectively ($P$<0.05). The effect of Aedae-NPF showed a large scatter among individual stomach preparations. At $10^{-6}$ mol $l^{-1}$, voltage reductions between 9% and 68% of the control value were measured. The effectiveness of the peptide showed no relation to the magnitude of $V_{te}$ under control conditions. The effect of the peptide was only partly reversible. However, it must be taken into consideration that in this kind of experiment the tissue is exposed to increasing concentrations of the modulator for 30–60 min, which certainly does not favor a successful washout.

In the next series of experiments ($N$=6) we tested whether proctolin (for a review, see Konopinska and Rosinski, 1999) affects $V_{te}$ of the anterior stomach of *A. aegypti* larvae. In all six experiments the individual tissues were subsequently exposed to hemolymph-side proctolin at increasing concentrations between $10^{-16}$ and $10^{-6}$ mol $l^{-1}$. The results are summarized in Fig. 4. Proctolin effected an almost continuous decrease of $V_{te}$ with increasing concentration. However, only at concentrations between $10^{-10}$ and $10^{-6}$ mol $l^{-1}$ did the results satisfy the criteria for statistically significant difference from the initial control value ($P$<0.05). As with Aedae-NPF, the voltage reduction induced by proctolin showed a large scatter (9–78% of the control at $10^{-6}$ mol $l^{-1}$), which was independent of the magnitude of $V_{te}$ before addition of the peptide. The effects of proctolin were only partly reversible.

### *Effects of neuropeptides on stomach motility*

Within 2 h after addition of serotonin (0.2 μmol $l^{-1}$), many preparations generated fluctuations of the transepithelial voltage ($\Delta V_{te}$) that were independent of the magnitude of $V_{te}$. At the same time when $\Delta V_{te}$ appeared, muscular motility of the mounted tissue could be observed through the preparation microscope. The motility included regular peristaltic waves. However, often it was observed to contain less regular contractions and seemed to reflect pump-like mixing of stomach contents instead of directional transport by peristaltic activity. With different preparations different patterns of $\Delta V_{te}$ were observed (see Fig. 5A). In some preparations, the muscular motility was reflected in a relatively regular sinusoidal wave. In others, their appearance resembled a double saw, where two waves of different frequency and

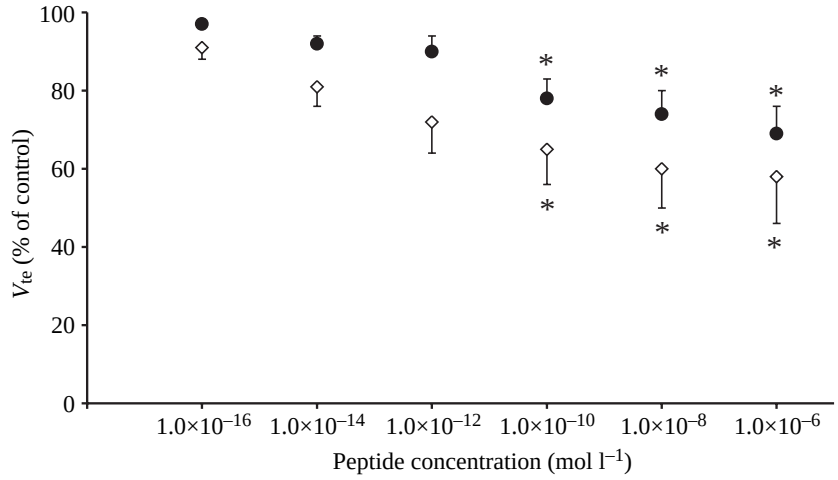


Fig. 4. Transepithelial voltages ($V_{te}$) expressed as percentage of the control voltage (100%) for different concentrations of Aedae-NPF (filled circles; control voltage –32±6 mV, $N$=9) and proctolin (open diamonds; control voltage –37±5 mV, $N$=6). Values are means + or – S.E.M. Asterisks indicate significant difference from the control ($P$<0.05).

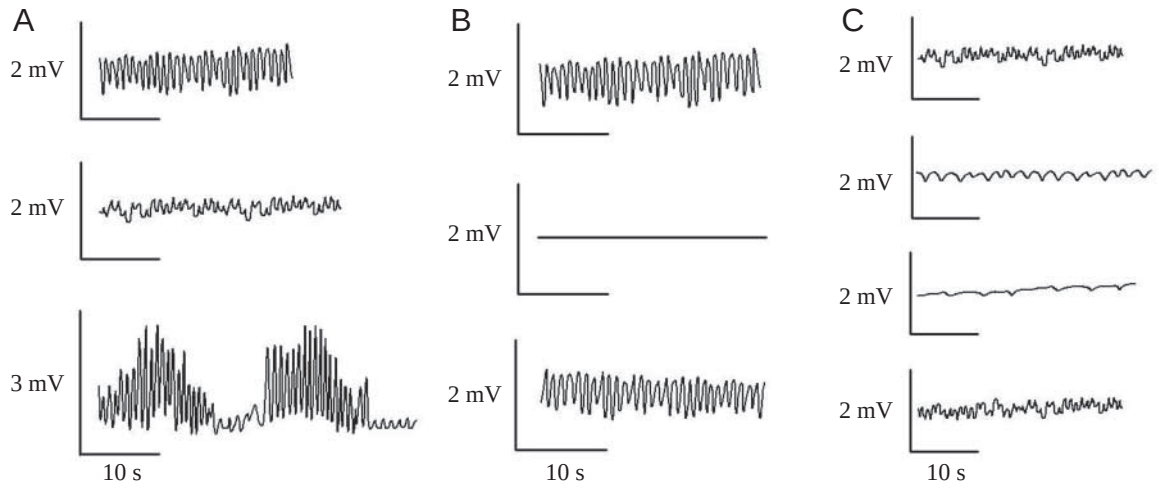


Fig. 5. (A) Examples for the different patterns of voltage fluctuations observed with isolated and perfused anterior stomach preparations of larval *A. aegypti*. (B) Representative example of the recordings of voltage fluctuations of the anterior stomach of *A. aegypti* larvae, showing their dependence on the presence of serotonin ($N$=7). Upper trace recorded in presence of serotonin (0.2 μmol $l^{-1}$), middle trace after its washout, and lower trace after re-establishing serotonin. (C) Representative example of the recordings of voltage fluctuations in absence of Aedae-HP-I and in presence of different concentrations of the peptide ($N$=5). Voltage traces from top to bottom: control, Aedae-HP-I $10^{-8}$ mol $l^{-1}$; Aedae-HP-I $10^{-6}$ mol $l^{-1}$; control after washout of Aedae-HP-I.

amplitude appeared to overlap. In some cases bursts of high wave activity were regularly interrupted by phases of much lower activity. Both $\Delta V_{te}$ and muscular motility were simultaneously and reversibly affected by washout of serotonin or by addition of peptides, indicating a direct relationship between $\Delta V_{te}$ and motility. In order to quantitatively assess $\Delta V_{te}$ the voltage signal was recorded at increased gain (0.5–1.0 mV $cm^{-1}$) and paper velocity (0.2 cm $s^{-1}$). The amplitude of the voltage oscillation was determined in 15 time intervals of 1 s and afterwards averaged. The frequencies were determined by counting oscillation peaks during a time period of 15 s. In nine experiments the preparations generated fluctuations of $V_{te}$ with an amplitude of 0.65±0.20 mV (mean ± S.E.M.) and frequency of 1.42±0.05 $s^{-1}$ (mean ± S.E.M.).

The $V_{te}$ fluctuations and the stomach motility are not instantaneously induced after the first addition of serotonin to the bathing medium. However, once initiated (60–120 min after addition of the drug) they were strictly dependent on the presence of serotonin (Fig. 5B). Washout of serotonin rapidly abolished $\Delta V_{te}$ and stomach motility, and re-addition of the drug re-established them ($N$=7).

Like Aedae-HP-I (see Fig. 5C), Aedae-HP-III, Aedae-sNPF and Aedae-NPF also reversibly inhibited $\Delta V_{te}$ and the stomach motility in a dose-dependent way. To quantify the influence of the neuropeptides, experiments were performed in which a peptide was applied at increasing concentrations ($10^{-8}$ and $10^{-6}$ mol $l^{-1}$) before and after recording $\Delta V_{te}$ under control conditions. Analyzing the amplitudes of $\Delta V_{te}$ under control conditions and comparing them with those measured in the presence of different concentrations of the neuropeptides

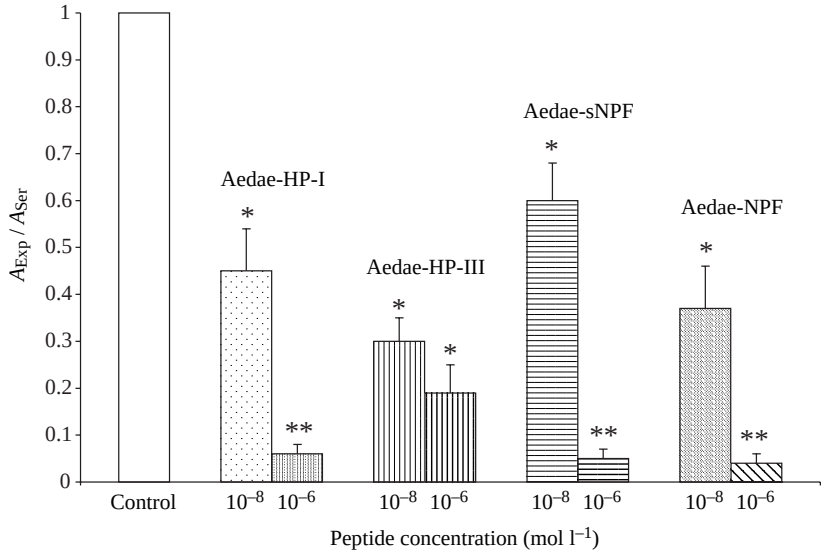


Fig. 6. Amplitudes of voltage fluctuations in presence of neuropeptides related to the control with 0.2 μmol $l^{-1}$ serotonin only ($A_{Exp}/A_{Ser}$). Aedae-HP-I (bars with dots, $N$=7, + S.E.M.), Aedae-HP-III (bars with vertical lines, $N$=7, + S.E.M.), Aedae-sNPF (bars with horizontal lines; $N$=5, + S.E.M.), Aedae-NPF (bars with diagonal lines, $N$=7, + S.E.M.). Each peptide was applied at $10^{-8}$ and $10^{-6}$ mol $l^{-1}$. *Significant difference from the control ($P$<0.05); **significant difference from the lower peptide concentration ($P$<0.05) in addition to the difference from the control.

clearly demonstrated the dose-dependent inhibitory effect of Aedae-HP-I, Aedae-sNPF and Aedae-NPF (see Fig. 6). For these peptides, the amplitudes of $\Delta V_{te}$ at $10^{-8}$ mol $l^{-1}$ were significantly lower than those under control conditions ($P<0.05$). At $10^{-6}$ mol $l^{-1}$ these peptides caused a further decrease of the amplitudes of $\Delta V_{te}$, which were found to be significantly lower than the amplitudes under control conditions and in presence of the lower neuropeptide concentration ($P<0.05$). Aedae-HP-III also caused a reduction of the amplitudes of $\Delta V_{te}$. However, in this case the two different peptide concentrations did not result in significantly different effects on the amplitude of $\Delta V_{te}$ (see Fig. 6), suggesting that Aedae-HP-III already exerts its maximal influence on stomach motility at $10^{-8}$ mol $l^{-1}$.

Analysing the influence of neuropeptides on the frequencies of $\Delta V_{te}$ ($N$=5–7) showed that this parameter is affected to a minor degree by the peptides. When compared with the frequencies under control conditions, the lower peptide concentrations ($10^{-8}$ mol $l^{-1}$) did not result in significant changes ($P>0.05$) of the frequencies. Only at $10^{-6}$ mol $l^{-1}$ did Aedae-HP-I (0.52±0.14 $s^{-1}$), Aedae-sNPF (0.33±0.13 $s^{-1}$) and Aedae-NPF (0.53±0.31 $s^{-1}$) significantly reduce the frequencies of $\Delta V_{te}$ ($P<0.05$) when compared with the control (1.47±0.04 $s^{-1}$).

The influences of Aedae-AST-A, Aedae-AT and proctolin on $\Delta V_{te}$ were not studied in detail. During the experiments with Aedae-AT voltage fluctuations were never observed. In one of the five experiments with the different Aedae-AST-A, $V_{te}$ fluctuations were observed and not inhibited by $10^{-7}$ mol $l^{-1}$ of the peptides. In one experiment with proctolin, $V_{te}$ fluctuations and motility were observed directly after administration of $10^{-16}$ mol $l^{-1}$ of the peptide; increasing the proctolin concentration did not further modify $\Delta V_{te}$. In the other five experiments with proctolin, no $\Delta V_{te}$ or motility were observed.

## Discussion

Based on the presence of a large number of endocrine cells, the insect midgut has been described as the largest endocrine organ (Brown et al., 1985). Its analogy to the gastroenteropancreatic endocrine system of mammals has been considered and various neuropeptides have been localized in this organ (cf. Sehnal and Zitnan, 1996). Thus, supplementing the neuropeptides liberated by the central nervous system, the enteric nervous system and gastrointestinal endocrine cells could substantially contribute to regulation of midgut functions, including ion transport. With the proceeding identification of neuropeptides in insects it becomes more and more important to gain insight into their specific effects and modes of action.

### *Methodological aspects*

The validity and advantages of the semi-open preparation of the isolated and perfused anterior stomach of larval mosquitoes have been demonstrated and discussed in detail in a previous study (Onken et al., 2004). With respect to the regulation of transport rates the qualitative aspect of the transepithelial voltage ($V_{te}$) is not ideal and the development of a technique to quantitatively monitor ion transport in the mosquito stomach remains a major task. Nevertheless, $V_{te}$ measurements are certainly useful for detecting changes induced by transport modulators, although their interpretation can be more complicated and insight into the mechanism of action of a modulator is difficult without simultaneous, area-specific conductance measurements. $V_{te}$ is determined by paracellular and transcellular parameters and a hormone-induced change of $V_{te}$ could reflect a change of the transport rates *via* an effect on the paracellular conductance, as has been proposed for the leucokinin-induced stimulation of fluid secretion by Malpighian tubules of *A. aegypti* (cf. Beyenbach, 2003; Yu and Beyenbach, 2004). When a modulator affects only the paracellular resistance the changes of $V_{te}$ and transport rates are opposite. Reduction of the paracellular resistance results in an increased transport rate at decreased $V_{te}$, whereas an increased paracellular resistance causes reduction of transport rates at increased $V_{te}$. However, the vast majority of hormonal modulators seems to act on electromotive force and/or conductance of the transcellular pathway, and in this case an increasing $V_{te}$ reflects transport stimulation and a decreasing $V_{te}$ reduction of the transport rate.

As in a previous study with the semi-open preparation of the larval anterior stomach of *A. aegypti* (Onken et al., 2004), $V_{te}$ under control conditions and after stimulation with serotonin showed considerable variability. Of course, we cannot rule out that this variation is partly based on different voltage decrements *via* the open ends of the preparations, reflecting a different longitudinal resistance of preparations of different length. Interestingly, however, the effects of neuropeptide F and proctolin on $V_{te}$ also showed a large scatter from preparation to preparation, and the effectiveness showed no relation to the magnitude of $V_{te}$ under control conditions before addition of the peptides (see Results). This observation suggests a significant variability between individual preparations that seems not to be related to the method. Instead, the differences may reflect a variability of the characteristics of the tissue from individual to individual. Such differences could be related, for example, to age and/or feeding behavior of fourth instar larvae and need further, more detailed studies.

During the present study, we noticed an additional feature of the preparation that is of particular importance for the evaluation of possible modulators of larval mosquito stomach functions. The stomach motility appears to be reflected in small, but properly recordable $V_{te}$ deflections with different patterns (see Fig. 5A). The dependence of $V_{te}$ fluctuations and of the observable stomach motility on the presence of serotonin and their inhibition by peptides (see below) is an indication that the dynamic $V_{te}$ component reflects muscular motility. In fact, the $V_{te}$ fluctuations cannot be related to the force of muscular activity, as is possible with other bioassays that use microforce transducers. We do not know how the motility generates fluctuations of $V_{te}$. It is certainly possible that the stomach motility could generate waves of small hydrostatic

pressure changes in the lumen that could in turn influence ion movement across ion-selective paracellular pathways. However, we cannot exclude direct effects of pressure gradients on the transcellular ion movement (Wang et al., 2003) or other explanations. More detailed experiments are needed to uncover the link between motility and $V_{te}$. Nevertheless, as the results of the present study indicate, the $V_{te}$ fluctuations seem to directly reflect muscular motility. Thus, the semi-open preparation of stomach segments could be an especially powerful tool for studying the effects of possible modulators on midgut function, because it seems to allow simultaneous monitoring of transepithelial ion transport (reflected in $V_{te}$) and stomach motility (reflected in its fluctuations).

### *Effects of peptides*

The number of gastrointestinal endocrine cells of insects, their degree of FMRFamide-immunoreactivity and the hemolymph concentration of the immunoreactive agent were observed to change as a response to feeding (Brown et al., 1986; Jenkins et al., 1989). For gastrointestinal functions like muscular motility (Schoofs et al., 1993), salivary gland fluid secretion (Duve et al., 1992), midgut secretion of digestive enzymes (Fusé et al., 1999; Nachman et al., 1997), fluid secretion by Malpighian tubules (Beyenbach, 2003; Coast et al., 2002) and reabsorption by the hindgut (Coast et al., 2002), regulation by peptides is well documented. In contrast, to our knowledge only a single study was performed that is related to the regulation of midgut ion transport by neuropeptides (Lee et al., 1998).

All effects of peptides on $V_{te}$ and its fluctuations observed in the present study appeared quickly after addition of the modulators to the bathing medium (see Fig. 1). In the experiments where effects of Aedae-NPF and proctolin on $V_{te}$ were observed the reversibility was limited, probably due to the long exposure of the tissue to the respective peptide. Interestingly, stomach motility and $V_{te}$ fluctuations appeared only 60–120 min after addition of serotonin. This observation could be explained if liberated endogenous peptides inhibit peristaltic activity until they are washed out.

### *Allatotropin and allatostatin*

Peptide factors of the nervous system are known to stimulate (allatotropin) or inhibit (allatostatin) the synthesis of juvenile hormone in the corpora allata. Both peptide types were also shown to have myotropic effects, including on gut peristalsis (Lange et al., 1993; Duve and Thorpe, 1994; Veenstra et al., 1994). Moreover, allatostatin was shown to stimulate midgut carbohydrate enzyme activity (Fusé et al., 1999) and allatotropin was demonstrated to inhibit ion transport across *Manduca sexta* posterior midgut (Lee et al., 1998). An Aedae-AT (Veenstra and Costes, 1999) and different Aedae-AST-A (Veenstra et al., 1997) have been isolated from adult *A. aegypti*. The identification of a partial prepro-allatostatin cDNA from a midgut cDNA library indicated that this gene is also expressed in the mosquito midgut (Veenstra et al., 1997). As shown in the present study (see Fig. 2), the five allatostatins acted in an almost identical way on $V_{te}$ of the anterior stomach, causing a slight but significant reduction. In contrast, Aedae-AT was without significant effect on $V_{te}$ in the whole concentration range studied (see Fig. 3).

Although the effects of Aedae-AT and Aedae-AST-A on stomach motility were not studied in detail, voltage fluctuations were never observed after addition of Aedae-AT. Thus, this peptide apparently does not induce muscular motility in the anterior stomach of *A. aegypti*. In one of the five experiments with the Aedae-AST-A, muscular contractions and $V_{te}$ fluctuations were observed and not inhibited by the peptides, suggesting that allatostatins do not inhibit motility in this tissue. Nevertheless, additional studies are needed to verify that Aedae-AT and Aedae-AST-A do not affect stomach motility in larval *A. aegypti*.

### *Head peptides and short neuropeptide F*

Different head peptides have been isolated from *A. aegypti* (Matsumoto et al., 1989; Veenstra, 1999). Antiserum against Aedae-HP-I reacted with gastrointestinal endocrine cells of adult mosquitoes (Brown et al., 1994) and the gene is expressed in larvae, adult males and adult females in response to a blood meal (Stracker et al., 2002). Aedae-HP-I was demonstrated to induce supression of host-seeking behaviour in adult females (Brown et al., 1994). A gene encoding short neuropeptide F (sNPF) has been found in *Drosophila melanogaster* and *Anopheles gambiae* (Riehle et al., 2002), and the Aedae-sNPF was isolated from an adult abdomen extract based on its immunoreactivity in a NPF radioimmunoassay. In the present study, Aedae-HP-I, Aedae-HP-III and Aedae-sNPF did not induce significant changes of $V_{te}$ across the anterior stomach of larval *A. aegypti* (see Fig. 3), indicating that these peptides do not interfere with ion transport *via* the serotonin-stimulated cells. However, all three peptides clearly inhibited stomach motility (see Figs 5C, 6). Aedae-HP-I and Aedae-sNPF supressed the amplitude of the $V_{te}$ fluctuations apparently more effectively than Aedae-HP-III. The finding of a myotropic effect of head peptide is contradictory to the results of a study on hindguts of adult *A. aegypti* where Aedae-HP-I was without effect on peristalsis. It could be, however, that muscular motility in different regions of the gut is modulated by different peptides.

### *Neuropeptide F*

Neuropeptide F (NPF), a member of the neuropeptide F/Y superfamily, was isolated from adult *A. aegypti* and NPF-like immunostaining was observed in brain and midgut of adults and larvae (Stanek et al., 2002). In adult females the NPF hemolymph concentration dropped immediately after a blood meal but showed a peak 24 h after the blood meal, suggesting a relation to digestive processes and the reproductive cycle (Stanek et al., 2002). In larvae, submicromolar concentrations of the peptide induced a decrease of $V_{te}$ across the anterior stomach (see Fig. 4). In addition, a strong inhibitory effect on stomach motility was observed (see Fig. 6). Thus, Aedae-NPF

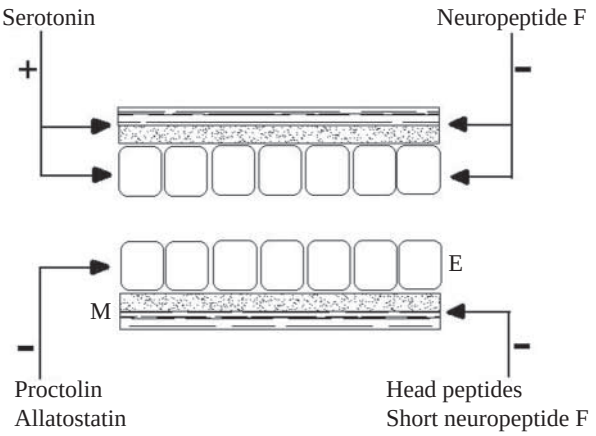


Fig. 7. Sketch summarizing the effects of neuropeptides on ion transport across the epithelial cell layer (E) and peristaltic activity of the muscle layer (M) of the anterior stomach of larval *A. aegypti*. + denotes stimulative and inhibitory effects on epithelial and/or muscle layers of the anterior stomach tissue.

is evidently involved in the regulation and coordination of two major functions of the anterior stomach of mosquito larvae: alkalization and motility.

### *Proctolin*

Proctolin is the best known and most investigated myotropic neuropeptide in insects, and it is regarded as the main neuromuscular transmitter/modulator in the gut of insects (Konopinska and Rosinski, 1999). In a variety of insects, proctolin has been shown to induce or stimulate muscle contractions, including gut peristalsis (Orchard et al., 1989). In the present study, proctolin induced a dose-dependent reduction of $V_{te}$ (see Fig. 4), indicating that it affects transepithelial ion transport *via* the serotonin-stimulated cells at submicromolar doses. Thus, besides the promotion of vitellogenesis in the cockroach oocyte (Goudey-Perriere et al., 1994), the present finding is one of the rare examples of proctolin affecting a non-excitable cell type.

In the present investigation, possible effects of proctolin on $V_{te}$ fluctuations, and thus stomach motility, were not studied in detail. In one experiment proctolin was observed to apparently induce $V_{te}$ fluctuations at $10^{-16}$ mol $l^{-1}$ and the $V_{te}$ fluctuations were not further modified when the concentration of the peptide was increased. However, this effect was not reproduced in the other five experiments. Thus, it appears that proctolin does not influence muscular motility in the anterior stomach of *A. aegypti*, but this observation needs verification in future. Nevertheless, it is noteworthy that in a comparative study (Messer and Brown, 1995) cricket hindguts responded in the expected way with a stimulation of peristalsis to proctolin, whereas with the hindguts of adult mosquitoes, proctolin did not affect peristalsis.

### *Effects of peptides related to their amino acid sequences*

The amino acid sequences of the peptides used in the present study (see Table 1) show clear similarities between Aedae-HP-I, Aedae-HP-II and Aedae-sNPF that cannot be neglected. In fact, there has been a proposal to name all peptides with the R(K)-$X_1$-R-$X_2$amide C-terminal motif as short NPFs (cf. Mertens et al., 2002). It might be that they all bind with different affinities to the same receptor to exercise their inhibitory effect on stomach motility. In *D. melanogaster*, different G-protein coupled receptors have been identified for short and long NPFs (Garczynski et al., 2002; Mertens et al., 2002; Feng et al., 2003). The same can be anticipated for *A. aegypti*, based on the observation that long NPF inhibited ion transport (Fig. 4), whereas head peptides/short NPF did not (Fig. 3). That the five Aedae-AST-A produced almost the same inhibitory effect on $V_{te}$ may also be attributable to their action on the same receptor. Among the three types of peptides that reduced $V_{te}$, similarities in their sequence can hardly be seen and it seems reasonable to assume that proctolin, Aedae-NPF and Aedae-AST-A use different receptors to unfold their action on ion transport. Since the effects of peptides on ion transport and motility were antagonistic to the effects of serotonin, it seems likely that the peptides interfere with the signal transduction pathways of the biogenic amine.

### *Effects on transport and/or motility*

The effects of serotonin and of neuropeptides observed in the present study are summarized in Fig. 7. Four groups of modulators can be distinguished: (1) stimulants of transepithelial transport and stomach motility (serotonin), (2) inhibitors of transepithelial transport and stomach motility (neuropeptide F), (3) inhibitors of transepithelial transport (proctolin and allatostatin) and (4) inhibitors of stomach motility (head peptides and short neuropeptide F). The identified modulators offer the possibility of an effective regulation and coordination of ion transport (alkalization) and muscular motility. However, it cannot of course be excluded that direct neuronal influence and further hormones/neuropeptides are also involved in the regulation and coordination of the anterior stomach functions.

Although the present study provides new insights into the regulation of the activity of the anterior stomach of larval *A. aegypti*, it has revealed even more gaps in our knowledge. We still have not identified the modulator (hormonal or non-hormonal) that re-establishes proper function of the cells with depolarizing basolateral membrane voltage (cf. Clark et al., 2000), which seem to be crucial for alkalization to pH 10–12 (see Introduction). Moreover, future studies need to determine the mode of action of the identified regulators, how their effects are relayed to the intracellular level and which transporters are affected. Another question to be addressed is where the peptides are liberated and which factors govern their liberation.

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