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INHIBITION OF MIDGUT ION TRANSPORT BY ALLATOTROPIN (Mas-AT) AND *MANDUCA* FLRFamides IN THE TOBACCO HORNWORM *MANDUCA SEXTA*

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Summary

Short-circuit current (I_{sc}) across the posterior midgut of day 2 fifth-instar tobacco hornworms (*Manduca sexta*) is inhibited by *Manduca* allatotropin (Mas-AT) and two *Manduca* FLRFamides (F7G and F7D). Another FLRFamide, F10, and the related molluscan tetrapeptide FMRFamide elicited only a modest inhibition of I_{sc}. Mas-AT, F7G and F7D inhibited the I_{sc} in a dose-dependent manner. Maximal inhibition of I_{sc} by Mas-AT was achieved at a concentration of 50 nmol l^{-1}. At 100 nmol l^{-1}, Mas-AT produced a significantly greater inhibition of I_{sc} than did F7G and F7D. The inhibition caused by Mas-AT was reversed by removing the peptide. Furthermore, the action of Mas-AT could be blocked by preabsorption with its antiserum. When F7G or F7D was added after the I_{sc} had been inhibited by Mas-AT, a further reduction in the I_{sc} was observed. No additive effects were seen when F7G and F7D were added together. In comparison with the anterior and middle regions, the posterior section of the midgut was the most sensitive to these three peptides. The I_{sc} of midguts dissected from feeding fourth instars was inhibited by Mas-AT, F7D and F7G, whereas the I_{sc} of midguts dissected from pharate fifth instars or wandering fifth instars was virtually unaffected by these peptides. Active ion transport across the posterior midgut of the silk moths *Hyalophora cecropia* and *Bombyx mori* was unaffected by these peptides.

Key words: *Manduca sexta*, tobacco hornworm, short-circuit current, FMRFamide, ion transport, midgut, allatotropin, neuropeptide.

Introduction

The midgut is an important site for both nutrient absorption and ionic regulation in lepidopteran larvae (Dow, 1986). The midgut epithelium of the tobacco hornworm *Manduca sexta* is composed of two major cell types, columnar and goblet cells (Cioffi, 1979). It is thought that the goblet cells are responsible for ion transport whereas the columnar cells are responsible for nutrient absorption (for reviews, see Klein *et al.* 1996; Sacchi and Wolfersberger, 1996; Turunen and Crailsheim, 1996). As larvae grow and develop, there are dramatic changes in the rate of active ion transport across the midgut. For example, during the molt between the fourth and fifth instars, there is a reduction in short-circuit current (I_{sc}), a measure of *in vitro* active ion transport (Chamberlin *et al.* 1997). After the molt, I_{sc} rises and remains high until the insect reaches the wandering stage. At this point in the insect's development, the larva is committed to pupation and there is an abrupt decline in midgut active ion transport (Chamberlin, 1994; Chamberlin and King, 1998). These developmental fluctuations in midgut ion transport could be controlled by the nervous system or by hormonal stimuli, but this aspect of lepidopteran midgut physiology has not been explored.

It is clear, however, that many neuropeptides, which are present in the neurosecretory cells of the central nervous system, have also been found in midgut endocrine cells or in the gut innervations of the enteric nervous system (for a review, see Sehnal and Zitnan, 1996). Among those neuropeptides, allatotropin (Mas-AT) immunoreactivity and mRNA have been identified in the nervous tissue during all stages of development and in the midgut of the larval stage of the tobacco hornworm (Zitnan *et al.* 1993, 1995*b*; Veenstra and Hagedorn, 1993; Veenstra *et al.* 1994; Taylor *et al.* 1996; Bhatt *et al.* 1997). Nevertheless, the role of Mas-AT during the larval stage is unclear because the only physiological roles of Mas-AT that have been identified are in the pharate adult and adult stages. These are the stimulation of juvenile hormone (JH) biosynthesis by the corpora allata (CA) of pharate adults and adult females (Kataoka *et al.* 1989; Hebda *et al.* 1994) and the stimulation of heart rate in the adult moth (Veenstra *et al.* 1994).

In addition to Mas-AT, three *Manduca* FLRFamides (F10, F7G and F7D; see Table 1 for amino acid sequences) have been identified in larval nervous tissue (Kingan *et al.* 1996) and larval midgut (Kingan *et al.* 1997). These peptides have been shown to affect muscle activity in the adult lepidopteran gut (Kingan *et al.* 1996; Fujisawa *et al.* 1993). Despite the presence of FLRFamides and Mas-AT-immunoreactive substances in the larval lepidopteran midgut, the role of these, or any other peptides, in modulating larval midgut function has not been

Table 1. *Amino acid sequences of the peptides used in this study of midgut ion transport*

Peptide	Amino acid sequence	Reference
Mas-AT	GFKNVEMMTARGF-NH_2	Kataoka *et al*. (1989)
F10	pEDVVHSFLRF-NH_2	Kingan *et al*. (1990)
F7G	GNSFLRF-NH_2	Kingan *et al*. (1996)
F7D	DPSFLRF-NH_2	Kingan *et al*. (1996)
FMRFamide	FMRF-NH_2	Price and Greenberg (1977)
Mas-AST	pEVRFRQCYFNPISCF-OH	Kramer *et al*. (1991)
Dip-AST7	APSGAQRLYGFGL-NH_2	Woodhead *et al*. (1989)

pE of F10 and Mas-AST is pyroglutamate.

explored. Peptide hormones have been shown to modulate ion transport in a variety of insect epithelia (for a review, see Gäde *et al.* 1997) and, Mas-AT or the FLRFamides may therefore have a similar function in the larval lepidopteran midgut. The present study examines, for the first time, the effects of several peptides (Mas-AT, F7G, F7D, F10 and others) on *in vitro* ion transport in the midgut of lepidopteran larvae.

Materials and methods

Insects

Manduca sexta L., originally derived from a colony of USDA (North Dakota) stock, were reared and staged as described by Chamberlin *et al.* (1997). Larvae were reared individually on a commercial diet (no. 9783, BioServ, Frenchtown, NJ, USA) at 25 °C on a 16 h:8 h light:dark cycle. The feeding fourth instars used in this study weighed between 0.9 and 1.4 g and showed no signs of preparation for molting (e.g. gut purging, head capsule slippage). Pharate fifth instars were selected as stage F larvae (black mandibles of the fifth instar visible through a clear slipped head capsule; Baldwin and Hakim, 1991). Stage F larvae were set aside 4–7 h before lights off. These larvae molted within a few hours and were designated day 0 in the next light cycle. For this study, feeding fifth instars, weighing between 3.5 and 5 g, were used on day 2. Wandering larvae were collected on day 5 from Gate II larvae (weighing less than 5 g on day 2; Chamberlin, 1994).

Giant silkworms (*Hyalophora cecropia*) were raised from eggs that had been collected in the field. The hatched larvae were reared in leaves of black cherry (*Prunus serotina*) at room temperature. Silkworm (*Bombyx mori*) larvae were provided by Dr Donald H. Dean (The Ohio State University).

Electrophysiological measurements

Short-circuit current (I_{sc}) and transepithelial potential difference (PD) were measured using the method described by Chamberlin *et al.* (1997). Midguts were dissected and mounted in modified Ussing chambers that had a 0.0636 cm^2 opening. Each half of the chamber contained 7 ml of saline (Chamberlin, 1994), which was continuously bubbled with 100 % oxygen. The PD was monitored using calomel electrodes connected to the Ussing chambers *via* agar bridges. The I_{sc} was passed through silver electrodes. The I_{sc} and PD were monitored using a World Precision Instruments DVC 1000 epithelial voltage clamp, and data were recorded with a computer-based acquisition program, Datacan V (Sable Systems, Inc., Version 1.0).

Effect of peptides on midgut I_{sc}

Synthetic Mas-AT and FMRFamide was purchased from Sigma Chemical Company (St Louis, MO, USA). *Manduca* allatostatin (Mas-AST) and cockroach allatostatin (Dip-AST7) were provided by Dr S. S. Tobe (University of Toronto, Canada). F10 was provided by Dr T. G. Kingan (University of California, Riverside, CA, USA) and F7G and F7D was supplied by Dr J. L. Witten (University of Wisconsin, Milwaukee, USA). Mas-AT was dissolved in a solution containing 50 mmol l^{-1} imidazole buffer and 1 mmol l^{-1} methionine (pH 7.2). Other peptides were dissolved in 50 % methanol with 2 mmol l^{-1} HCl. Stock solutions of peptides were diluted prior to use in saline.

Posterior midguts from day 2 fifth instars were mounted in Ussing chambers and the I_{sc} was monitored for approximately 1 h. The voltage clamp was then turned off and the PD recorded 5 min later. After the PD measurement, the voltage clamp was turned on again, and 5 min later 10 µl of the peptide solution was added to the hemolymph side of the tissue. The I_{sc} was then monitored for 5 min, after which the voltage clamp was turned off again and the PD recorded. Resistance was calculated from the PD and I_{sc} measured before and after the addition of peptide. In some experiments, two different peptides were added sequentially to the hemolymph side of the posterior midgut to determine whether the different peptides exhibited additive or synergistic effects on the posterior midgut. To determine whether there were regional differences in the responses to these peptides, the effects of Mas-AT, F7G and F7D on the I_{sc} of the anterior and middle midgut sections were tested.

Effect of preabsorption of Mas-AT with its antiserum

Mas-AT was diluted 50-fold with its primary antiserum (provided by Dr S. J. Kramer, Sandoz Agro Inc., USA) in imidazole buffer. The mixture was incubated for 3 days at 5 °C while being continuously stirred. Mas-AT, without antiserum (control), was incubated under the same conditions. The effect of preabsorbed Mas-AT or the control solution on posterior midgut I_{sc} was determined as described above.

To confirm that the Mas-AT employed in this study was biologically active and that this activity could be blocked by preabsorption by its antiserum, stimulation of corpora allata

(CA) juvenile hormone (JH) biosynthesis by this peptide was measured using the *in vitro* radiochemical assay of Pratt and Tobe (1974), as modified by Feyereisen and Tobe (1981). A pair of CA was dissected from adult females and transferred into 100 µl of medium-199 (Gibco) with 20 mmol l^{-1} Hepes and 2 % Ficoll (Sigma, St Louis, MO, USA) containing L-[^{3}H-methyl]methionine (NEN, Boston, MA, USA) at a final concentration of 0.1 mmol l^{-1} (final specific activity of 3700 mBq mmol^{-1}). Corpora allata were incubated for 3 h at 30 °C with or without Mas-AT (20 nmol l^{-1}) or Mas-AT preabsorbed to antiserum. After incubation, 250 µl of iso-octane was mixed with the incubation medium and the mixture was centrifuged at 2500 ***g*** for 5 min. A 200 µl sample of the iso-octane (top) phase was then removed, mixed with 1 ml of distilled water and centrifuged at 2500 ***g*** for 5 min. A 150 µl sample of the top iso-octane phase was removed and its radioactivity determined by liquid scintillation counting (EcoLume, ICN, USA).

Effect of peptides on the I_{sc} of different developmental stages of Manduca sexta *and terminal larval instars of other Lepidoptera*

The effects of Mas-AT, F7G and F7D on the I_{sc} of the posterior midgut of feeding fourth instars, pharate fifth instars, day 2 fifth instars and wandering (day 5) fifth instars were compared. In addition, the posterior midguts of *H. cecropia* and *B. mori* were mounted in Ussing chambers and bathed in *Manduca* saline. The effects of Mas-AT, F7G and F7D on the I_{sc} of these epithelia were determined as described above.

Statistics

Values are expressed as means ± standard error of the mean (S.E.M.) with *N* indicating the number of midguts measured. Statistical analyses were conducted using an analysis of variance (ANOVA) and a Tukey's *post-hoc* test. When appropriate, paired analyses were conducted. Data presented as percentages were log-transformed before statistical analyses. $P \leqslant 0.05$ was considered to represent a significant difference.

Results

Figs 1 and 2 show some responses of the posterior midgut to Mas-AT and FLRFamides when the I_{sc} was not interrupted for PD measurements. Addition of Mas-AT (50 nmol l^{-1}) to the hemolymph side of the tissue promptly inhibited the I_{sc}, and maximal inhibition was reached within 5 min. The I_{sc} then recovered slowly (Fig. 1A). The inhibition of I_{sc} by Mas-AT was quickly reversed by rinsing the chambers with peptide-free saline (Fig. 1B). Addition of *Manduca* FLRFamide peptides, F7G and F7D (100 nmol l^{-1}), to the hemolymph side of the tissue also rapidly inhibited the I_{sc} (Fig. 2A,B), but the inhibition of I_{sc} by these two peptides was maintained for more than 1 h after treatment. Addition of all of these peptides to the lumen side of the midgut did not affect the I_{sc} (data not shown).

Inhibition of I_{sc} by Mas-AT, F7G and F7D was dose-

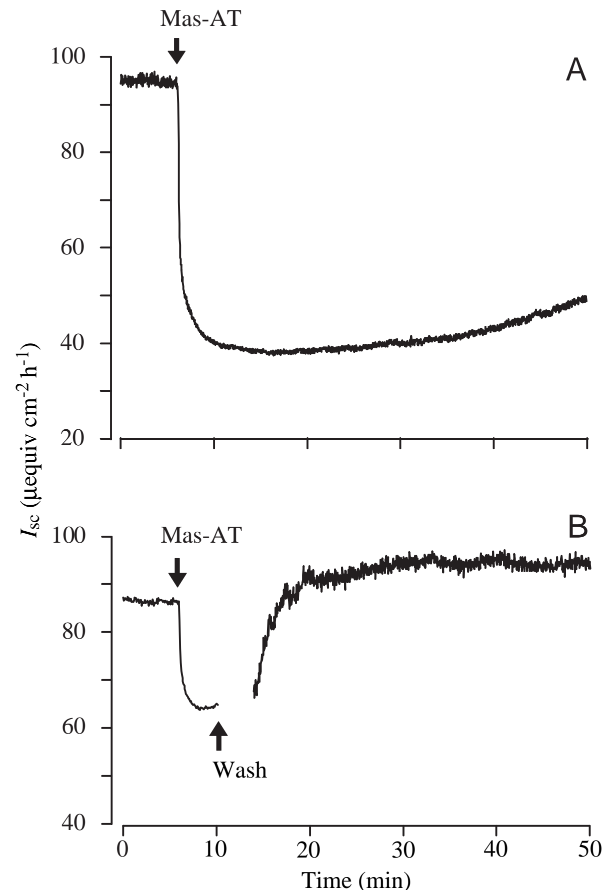

Fig. 1. Representative recordings showing the effects of the addition and removal of Mas-AT on the short-circuit current (I_{sc}) of the posterior midgut of fifth-instar *Manduca sexta.* (A) Mas-AT (final concentration 50 nmol l^{-1}) was added to the hemolymph side of the chamber. (B) When I_{sc} had been maximally inhibited by Mas-AT, the voltage clamp was turned off (indicated by a break in the recording) and the saline was replaced with peptide-free (Wash) saline. The voltage clamp was then turned on and the I_{sc} monitored.

dependent (Fig. 3), and all three peptides inhibited the I_{sc} at doses higher than 1 nmol l^{-1}. Maximal inhibition of I_{sc} by Mas-AT was achieved at 50 nmol l^{-1}, whereas F7G and F7D inhibited the I_{sc} maximally at 10 nmol l^{-1}. At 100 nmol l^{-1}, Mas-AT produced a significantly greater inhibition of I_{sc} than did F7D or F7G (Fig. 3). All three peptides significantly increased the resistance of the epithelium (Table 2). Addition of another *Manduca* FLRFamide, F10, or the molluscan tetrapeptide FMRFamide only slightly inhibited I_{sc} (7.9±1.3 %, *N*=7; 3.4±0.6 %, *N*=7, respectively, at 1 µmol l^{-1}; Fig. 3). Addition of other peptides, *Manduca* allatostatin (Mas-AST) and a cockroach (*Diploptera punctata*) allatostatin (Dip-AST7) did not change the I_{sc} when added at concentrations of 10–100 nmol l^{-1} (data not shown).

Mas-AT that had been preabsorbed with its antiserum failed to inhibit the I_{sc} (Fig. 4A). This was not due to non-specific damage to Mas-AT during the preabsorption process because Mas-AT that had been incubated in the absence of antiserum

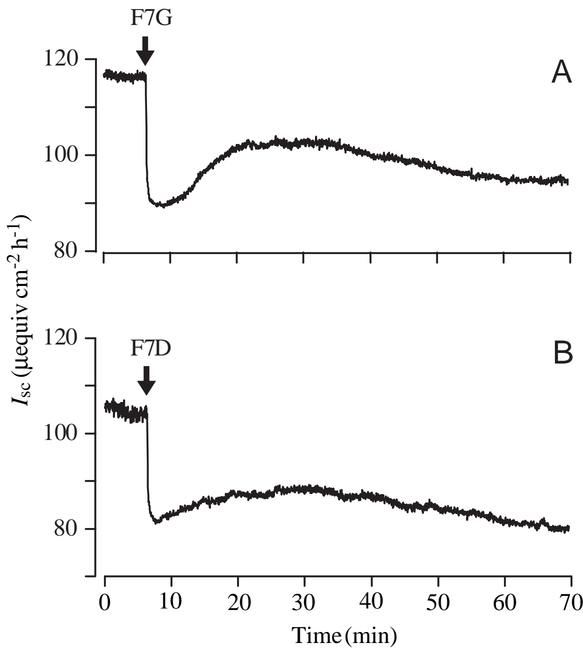

Fig. 2. Representative recordings showing the effects of (A) 100 nmol l^{-1} F7G and (B) 100 nmol l^{-1} F7D on the short-circuit current (I_{sc}) of the posterior midgut of day 2 fifth-instar *Manduca sexta*. Peptides were added at the points indicated by the arrows.

did inhibit the I_{sc} (Fig. 4B). Confirmation that preabsorption of Mas-AT with its antiserum abolishes its biological activity was obtained using an *in vitro* radiochemical assay of JH biosynthesis by CA. Total JH production by CA from day 3 adult females was 4.3±0.3 pmol h^{-1} CA pair^{-1} (N=4). When Mas-AT (20 nmol l^{-1}) was added to the incubation medium, this rate increased significantly to 22.7±3.6 pmol h^{-1} CA pair^{-1} (N=4). When preabsorbed Mas-AT was added, the rate of production of JH was only 4.5±1.7 pmol h^{-1} CA pair^{-1} (N=4). Thus, preabsorption with antiserum abolished the effects of Mas-AT in both the midgut and the CA.

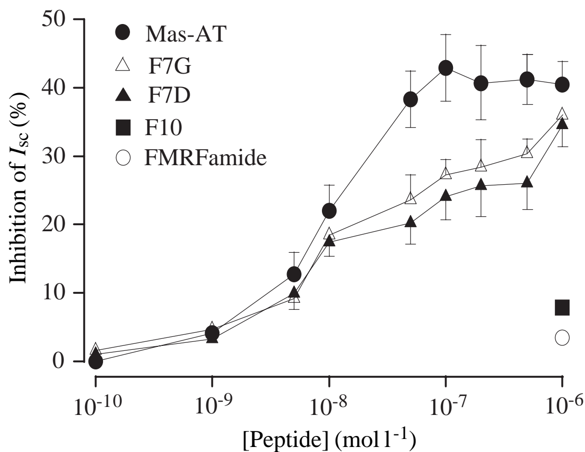

Fig. 3. Dose–response plot showing the effects of peptides on the short-circuit current (I_{sc}) of the posterior midgut of day 2 fifth-instar *Manduca sexta*. Values are means ± S.E.M. (N=5–7).

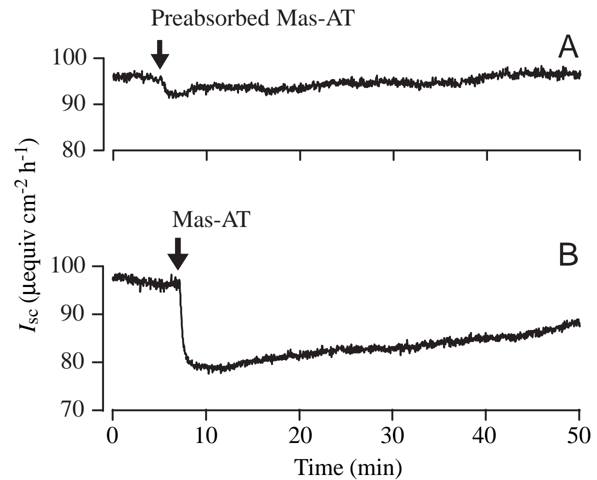

Fig. 4. Representative recordings showing the effects of preabsorbed Mas-AT on the short-circuit current (I_{sc}) of the posterior midgut of day 2 fifth-instar *Manduca sexta*. (A) Mas-AT (20 nmol l^{-1}) incubated for 3 days with its antiserum. (B) Mas-AT incubated for 3 days without its antiserum. Similar results were obtained in two additional experiments.

After the I_{sc} had been reduced by a saturating concentration (200 nmol l^{-1}) of Mas-AT, subsequent addition of Mas-AT did not significantly reduce the I_{sc} (Fig. 5A). Addition of F7G or F7D, however, significantly inhibited the I_{sc} (Table 3; Fig. 5A,C). Reversing the order of peptide exposure also produced additive inhibition of the I_{sc} (Fig. 5B,D; Table 3). The effects of F7G and F7D did not appear to be additive (Fig. 5E; Table 3).

The effects of Mas-AT, F7G and F7D on I_{sc} were determined in different midgut regions of day 2 fifth instars. Under control conditions, the I_{sc} of the anterior region (149.6±11.0 μequiv cm^{-2} h^{-1}, N=7) was significantly higher than that of the middle (99.1±9.8 μequiv cm^{-2} h^{-1}, N=7) and posterior (Table 2) regions. The greatest peptide-induced inhibition of the I_{sc}, however, occurred in the posterior region (Fig. 6A). Of the three peptides tested, Mas-AT produced a significantly greater inhibition of I_{sc} than F7G and F7D in all three regions of the midgut. The effects of these three peptides on posterior midguts of larvae at different stages of development were also determined (Fig. 6B). The control I_{sc} was highest in midguts dissected from the feeding stages of fourth (95.4±16.4 μequiv cm^{-2} h^{-1}, N=7) and fifth (Table 2) instars and lowest in those of molting larvae (23.8±5.6 μequiv cm^{-2} h^{-1}, N=6) and wandering larvae (12.6±2.0 μequiv cm^{-2} h^{-1}, N=6). Addition of Mas-AT, F7G or F7D caused the greatest inhibition of I_{sc} in feeding day 2 fifth instars, but minimal inhibition was observed in pharate fifth instars or wandering larvae. In the presence of Mas-AT, the lowest I_{sc} of the posterior midgut of feeding larvae was 47.3±5.9 μequiv cm^{-2} h^{-1} (N=6). This value is still significantly higher than the control I_{sc} of molting or wandering larvae.

When the posterior midguts of two other lepidopteran species were exposed to Mas-AT, F7G and F7D, the effects on

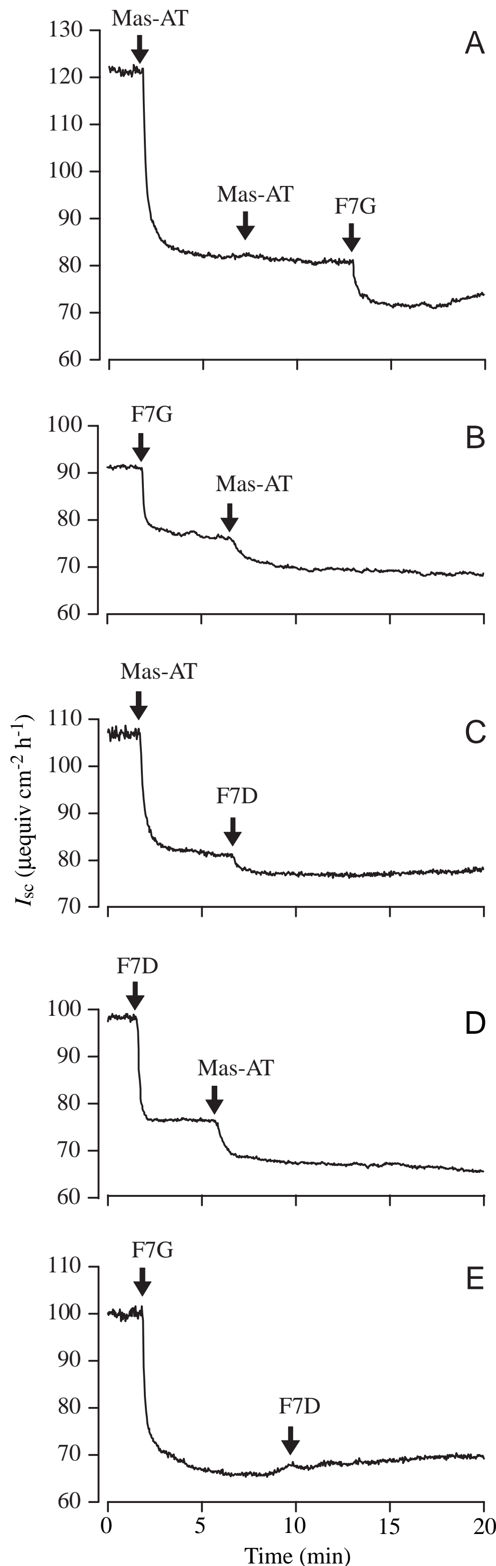

Fig. 5. Representative recordings showing the effects of sequential additions of peptides on the short-circuit current (I_{sc}) of the posterior midgut of day 2 fifth-instar *Manduca sexta*. (A) Two additions of Mas-AT followed by F7G. (B) Addition of F7G followed by Mas-AT. (C) Addition of Mas-AT followed by F7D. (D) Addition of F7D followed by Mas-AT. (E) Addition of F7G followed by F7D. Peptide concentrations were 200 nmol l^{-1}.

I_{sc} were quite different from those observed in *Manduca sexta*. Although the control I_{sc} values of *H. cecropia* (96.5±4.9 μequiv cm^{-2} h^{-1}, *N*=7) and *B. mori* (74.3±5.4 μequiv cm^{-2} h^{-1}, *N*=7) were quite high when bathed in *Manduca* saline, Mas-AT only inhibited the I_{sc} of *H. cecropia* and *B. mori* midguts by 5.4±1.3 % (250 nmol l^{-1}) and 3.9±0.8 % (100 nmol l^{-1}), respectively. Furthermore, addition of 100 nmol l^{-1} F7G and F7D did not affect the I_{sc} of the midguts of these two species.

Discussion

Allatotropin (Mas-AT) has previously been shown to stimulate JH biosynthesis by the CA (Kataoka *et al.* 1989) and to accelerate heart activity (Veenstra *et al.* 1994). Both these effects are restricted to the pharate adult and adult stages of development. No effect of this peptide has been detected in larvae (Kataoka *et al.* 1989; Unni *et al.* 1991; Hebda *et al.* 1994; Veenstra *et al.* 1994). Nonetheless, Mas-AT mRNA and immunoreactivity have been detected in both the larval and adult stages (Zitnan *et al.* 1993, 1995*b*; Veenstra *et al.* 1994; Taylor *et al.* 1996; Bhatt and Horodyski, 1998), suggesting that Mas-AT may have a biological role in larval insects that differs from its role in the adult. In the present investigation, it was demonstrated that the posterior midgut of feeding *M. sexta* larvae is a target for Mas-AT action. The peptide rapidly inhibits *in vitro* active ion transport across the midgut epithelium. Furthermore, two *Manduca* FLRFamides, F7G and F7D, elicit a similar rapid inhibition of ion transport in the same tissue. Both F7G and F7D are members of a larger family of RFamide peptides characterized by a common C terminus (Kingan *et al.* 1996). Although F10 is similar to F7G and F7D and shares a common C terminus with these peptides, it did not inhibit midgut I_{sc}. This difference in biological activity may be due to differences in the structures of the peptides in saline. In solution, F7G and F7D do not exhibit a secondary structure, whereas F10 does (T. G. Kingan, personal communication). This altered conformation may be responsible for the lack of activity of F10 in the present study. In adult *M. sexta*, FLRFamides exhibit myostimulatory activities on the skeletal flight muscles (F10; Kingan *et al.* 1990) and on the ileum (F7G and F7D; Kingan *et al.* 1996). Although F10 was shown to have myosuppressive activity on the adult midgut of a related sphingid moth, *Agrius convolvuli* (Fujisawa *et al.* 1993), the larval midgut represents a novel target of FLRFamides (Zitnan *et al.* 1993, 1995*a*; Kingan *et al.* 1997). It is not known whether Mas-AT or FLRFamides exert myostimulatory or myosuppressive actions on the larval midgut of *M. sexta*, but it is clear from the present study and other investigations that Mas-AT, F7G and F7D have multiple physiological activities in different tissues during distinct developmental stages. The inhibition of I_{sc} by both Mas-AT and the FLRFamides was rapid, and the inhibition due to Mas-AT was reversible. The response of the midgut

Table 2. *Effect of Mas-AT, F7G and F7D on the short-circuit current, transepithelial potential difference and resistance of the posterior midgut of day 2 fifth-instar* Manduca sexta

Peptide	I_{sc} (μequiv cm^{-2} h^{-1}) Before	I_{sc} After	PD (mV) Before	PD After	Resistance (Ω cm^2) Before	Resistance After
Mas-AT	99.4±5.3	58.3±5.0*	76.1±4.7	75.0±4.4	28.8±1.8	49.4±3.4*
F7G	99.9±7.2	77.8±9.1*	80.9±3.1	79.8±3.6	31.0±2.1	41.2±4.5*
F7D	96.1±6.4	77.0±5.8*	79.8±3.8	77.7±3.9*	31.8±2.5	38.8±3.3*

Values are means ± S.E.M. (*N*=6–7).
*A significant difference ($P<0.05$) from the value before the addition of peptide (50 nmol l^{-1}).
T_{sc}, short-circuit current; PD, transepithelial potential difference.

to these peptides was dose-dependent, and the peptide concentrations required for activity are similar to those required in other target tissues (Kataoka *et al.* 1989; Kingan *et al.* 1996; Veenstra *et al.* 1994).

Although Mas-AT and the FLRFamides F7G and F7D inhibited I_{sc} in the posterior midgut, the only structural feature these peptides have in common is the Famide in the C terminus. It is unlikely that this structure is the sole determinant of biological activity, since F10, which also shares this structure (see Table 1), is physiologically inactive on the larval posterior midgut. In addition, the actions of Mas-AT and the FLRFamides are additive, indicating that these peptides act on different cells within the midgut and/or through different receptors on the same cells. The specific cellular target(s) of these peptides is not known. Nevertheless, the sensitivity of the posterior midgut to these peptides points to a target or targets specific to this region of the midgut. The columnar and goblet cells of the posterior midgut have a different morphology from those in the anterior and middle regions (Cioffi, 1979) and, therefore, either cell type could be the target of these peptides. It seems likely, however, that the inhibition of I_{sc} by these peptides is due to a direct action on the goblet cells because it is this cell type that is thought to be responsible for transepithelial active ion transport (Klein *et al.* 1996). It is also possible that F7G or F7D acts primarily on midgut muscle cells since muscle cells have been shown to be targets of these peptides (Kingan *et al.* 1996). Modulation of muscle contraction may then have a secondary effect on the ion-transporting epithelial cells by changing the shape or surface area of the epithelium. Although the exact target(s) of Mas-AT, F7D and F7G has not been identified, it is also possible that the additive effects of these peptides is due to binding to distinct receptors on the basolateral membranes of the same cells. Support for the existence of distinct receptors for these peptides is provided by the observation that, in the adult CA, Mas-AT stimulates JH biosynthesis, whereas F7G and F7D do not (K.-Y. Lee, F. M. Horodyski and M. E. Chamberlin, unpublished observation).

The inhibitory actions of Mas-AT and FLRFamides appear to be species-specific. These peptides exert little or no inhibition of active ion transport across the posterior midgut of the silkmoths *H. cecropia* and *B. mori*. In contrast, it has been suggested that Mas-AT stimulates JH biosynthesis by the CA of Lepidoptera because it is effective in both *M. sexta* and *Heliothis virescens* (Kataoka *et al.* 1989). Additional studies on more lepidopteran species will need to be conducted before it can be concluded that the activity of Mas-AT on the midgut is truly restricted to *M. sexta* or whether its effect on the CA is widespread in the order.

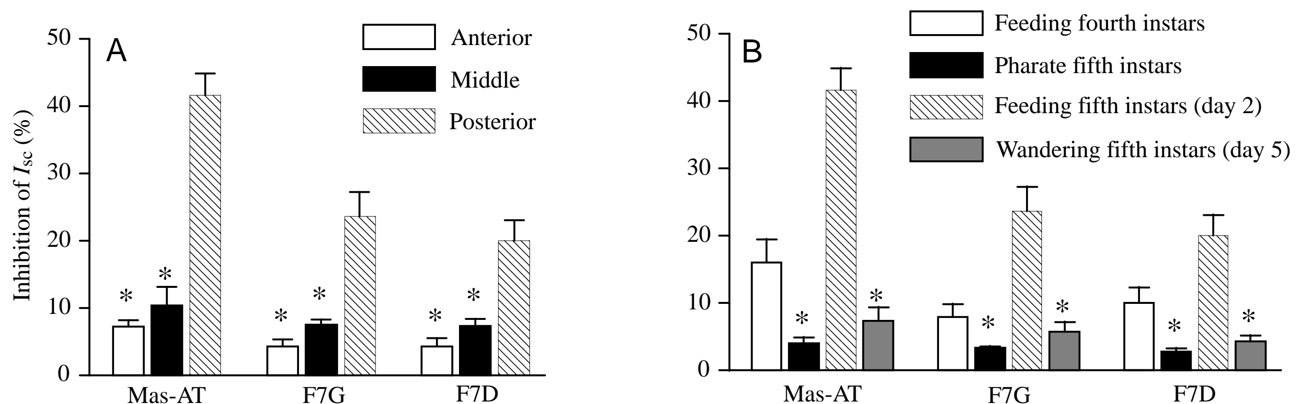

Fig. 6. Effects of (50 nmol l^{-1}) Mas-AT, F7G and F7D on the short-circuit current (I_{sc}) of (A) different regions of the midgut of day 2 fifth instars and (B) the posterior midgut of different larval stages of *Manduca sexta*. Values are expressed as means + S.E.M. (*N*=6–7). An asterisk denotes a value significantly different from that in (A) the posterior midgut or (B) the feeding fifth instar.

Table 3. *Additive effects of Mas-AT, F7G and F7D on the short-circuit current of the posterior midgut of day 2 fifth-instar* Manduca sexta

First peptide	I_{sc} (µequiv cm^{-2} h^{-1})	Second peptide	I_{sc} (µequiv cm^{-2} h^{-1})
Mas-AT	78.0±8.1	F7G	68.8±6.7*
F7G	79.8±9.9	Mas-AT	71.6±9.4*
Mas-AT	78.0±5.8	F7D	70.6±5.6*
F7D	78.0±14.1	Mas-AT	70.0±14.4*
F7G	84.1±12.4	F7D	84.6±12.8

I_{sc}, short-circuit current after the sequential addition of 200 nmol l^{-1} peptide.

Values are expressed as means ± S.E.M. (*N*=3).

*A significant decrease (P<0.05) in I_{sc} upon the addition of the second peptide.

It is not known whether the peptides that affect midgut active ion transport act as hormones or paracrines. The distribution of these peptides within the insect can provide clues to the source and the means by which they act on the target tissues. In the larva, Mas-AT mRNA is present at highest levels in the frontal ganglion and terminal abdominal ganglion (Taylor *et al.* 1996; Bhatt and Horodyski, 1998). Because the frontal ganglion cells project their axons down the recurrent nerve and innervate muscles at the foregut–midgut boundary (Copenhaver and Taghert, 1991), these cells may be the source of Mas-AT that acts as a paracrine on the posterior midgut. The Mas-AT synthesized in the other portions of the central nervous system may be released into the hemolymph and act as a hormone on the midgut. Because the gene for F7G and F7D has not been isolated, the cells in the nervous system that contain mRNA encoding these peptides have not been identified.

In addition to the cells that contain Mas-AT mRNA, other cells of the enteric nervous system and the midgut exhibit immunoreactivity against antiserum to Mas-AT (Zitnan *et al.* 1993). These include intrinsic neurons of the enteric plexus and the midgut endocrine cells. The presence of Mas-AT mRNA in these cells has not yet been investigated, so it is not possible to conclude whether these cells contain Mas-AT or a peptide that cross reacts with the antiserum. If Mas-AT is indeed present in these cells, then they could be a source of peptide for a paracrine action on nearby cells, which may include the ion-transporting cells of the posterior midgut. Direct support for this hypothesis will require the demonstration that the immunoreactive material is identical to Mas-AT. Recently Kingan *et al.* (1997) showed that F10, F7G and F7D are present in the larval midgut, and this finding indicates a potential paracrine role for FLRFamides.

During larval growth and development, the rate of midgut active ion transport changes dramatically. Previous studies (Chamberlin, 1994; Chamberlin *et al.* 1997; Chamberlin and King, 1998) have shown that *in vitro* active ion transport activity is high during the feeding stages of the fourth and fifth instars, but declines during the molting or wandering stages. The present study confirms these earlier findings and shows that Mas-AT and two FLRFamides greatly inhibited the I_{sc} in the midguts of feeding fourth and fifth instars, but that the I_{sc} of midguts dissected from molting or wandering larvae was only minimally affected by these peptides. Although these peptides inhibit the I_{sc} of midguts from feeding larvae, the inhibited I_{sc} is not as low as the control I_{sc} observed in molting or wandering larvae. Therefore, even if these peptides are involved in the suppression of active ion transport during molting and metamorphosis, it is clear that other processes are involved in reducing midgut ion transport during larval–larval molting and wandering. The relative insensitivity of the midguts of molting and wandering larvae to these peptides may reflect a loss of receptors during these periods. It is also possible that during larval–larval molting, when new midgut cells are formed (Baldwin and Hakim, 1991), the epithelium contains a large population of cells that have yet to express the receptor(s).

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