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Mechanisms and control of secretion in the Malpighian tubules of *Tenebrio molitor*: an immunohistochemical and electrophysiological study

by

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Submitted in fulfillment of the requirements for the degree of Doctor of Philosophy

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November 2002

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ACKNOWLEDGEMENTS

Looking back over the past three years, I am both happy and sad for the same reason…it is all over! Yes, it required dedication and hard work, of which I enjoyed every minute, but hard work alone could never have amounted to this. I had the help and support of some of the finest people and it's these people that I would now like to thank.

I would have to start off by thanking my supervisor, Prof. Sue Nicolson. It all started when I walked into your laboratory three years ago without the required qualifications or the background, but you never doubted my abilities. Thank you for your trust and for haven given me this great opportunity. Although most of our communications were long distance, I could have never wished for a better supervisor.

Special thanks goes to my co-supervisor, Prof. Emmy van Kerkhove (Limburgs Universitair Centrum, Diepenbeek, Belgium). You not only taught me everything I needed to know about the physiology of epithelia, but you made this foreigner feel right at home in your laboratory. Thank you for all those numerous discussions and constructive suggestions. It certainly improved the quality of this work.

I have never worked with people that were so willing to help and so easy to get along with than the staff members and the students of the Physiology department (L.U.C.). I'd like to extend my gratitude to Prof. Steels for all the hours he spend discussing potassium channels and taking time to answer all my questions. Kathleen, Josette, Patrick, Roland and Wilfried, thank you so much for the efficient way you attended to all my administrative/experimental needs. Thank you Sara, Bert, Ilse and Georg for the sometimes lighthearted and sometimes serious discussions around the coffee table. You were not only colleagues you became friends. Sara, I will never forget all you have done for me. Thank you.

To Prof. Lilliane Schoofs (Catholic University Leuven), many thanks for the opportunity to work in your laboratory. I would also like to thank the technical staff of KULeuven, Luc and Johnny as well as Maria for their assistance.

Lastly, I would like to thank family and friends for their moral support. Being abroad for such long periods of time really made me realize the value of my parents. Thank you mom and dad for those countless emails. They kept me going when the going got tough. I hope I did you proud.

My dear husband, I left you till the very end, mostly because I don't really know how to express my gratitude to you. When we took this decision, we could never imagine the strain it would put on our marriage, but we saw it through. Thank you for the unselfish way in which you supported me throughout this time. I am confident that after these three years we can accomplish anything together.

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LIST OF FREQUENTLY USED ABBREVIATIONS

ADF	antidiuretic factor
BSA	bovine serum albumin
CAP_{2b}	cardioacceleratory peptide 2b
CRF	corticotropin releasing factor
DH	diuretic hormone
DP	diuretic peptide
EIA	enzyme-linked immunoassay
ESI	electrospray ionization
FITC	fluorescein isothiocyanate
FMOC	fluorenylmethoxycarbonyl
IHC	immunohistochemistry
IIPO	indirect immunoperoxidase
ITP	Ion transport peptide
MALDI-TOF	matrix-assisted laser desorption ionization time of flight
NCC I	nervi corporis cardiaci I
NPS	normal porcine serum
NO	nitric oxide
PBS	phosphate-buffered saline
PIG	pre-immune goat
PPD	para-phenylenediamine
RPLC	reverse-phase liquid chromatography
SEM	standard error of the mean
SOG	suboesophageal ganglion
TBS	TRIS-buffered saline
TmPCP	*Tenebrio molitor* putative cuticle protein
V_{te}	transepithelial membrane potential
V_{ap}	apical membrane potential
V_{bl}	basolateral membrane potential

ABSTRACT

Fluid secretion by insect Malpighian tubules is controlled by haemolymph-borne factors. Two corticotropin-releasing-factor (CRF)-related diuretic peptides, Tenmo-DH_{37} and Tenmo-DH_{47}, previously isolated from *Tenebrio molitor*, were found to stimulate *in vitro* tubule preparations of *Tenebrio molitor via* the second messenger cyclic AMP. The stimulatory effect of Tenmo-DH_{37} was reversed on addition of endogenous antidiuretic peptides (Tenmo-ADFa and ADFb) and exogenous cardioacceleratory peptide 2b (CAP_{2b}), both acting *via* the second messenger cyclic GMP. The immunocytochemical localization of Tenmo-DH_{37} and the second antidiuretic peptide isolated from *Tenebrio molitor*, Tenmo-ADFb, was investigated using antisera raised against these hormones. Neurosecretory cells immunoreactive to Tenmo-DH_{37} were found in the brain and abdominal ganglia with immunoreactive processes projecting to the peripheral nervous system. Intense staining of the neurohaemal release site, the corpora cardiaca, was observed. In addition, neurosecretory cells immunoreactive to Tenmo-DH_{37} were found in the posterior midgut and a network of immunoreactive nerve processes extended over the surface of the midgut. Tenmo-ADFb immunoreactivity was localized in the brain, in two pairs of bilaterally symmetrical cells in the protocerebrum. *Tenebrio* tubule secretion appears entirely dependent on the surrounding K^+ concentration and intracellular measurements of the basolateral (V_{bl}) and indirectly apical membrane potentials (V_{ap}) indicate an appreciable sensitivity of both membranes to the bath K^+ concentration, but not to Na^+. Secretion assay and electrophysiological results indicate that K^+ uptake across the basolateral membrane is primarily through barium-sensitive K^+ channels, but also implicate a bumetanide-sensitive $Na^+/K^+/2Cl$ cotransporter, an ouabain-sensitive Na^+/K^+-ATPase and glibenclamide-sensitive K_{ATP} channels. Furthermore, electrophysiological evidence suggests that fluid secretion/inhibition by endogenous factors is achieved by influencing at least three parameters simultaneously: the rate of H^+ extrusion by the V-ATPase, basolateral K^+ conductance, and possibly Cl^- conductance. The effect of amiloride on fluid secretion and pH indicates the presence of a cation/H^+ exchanger in Malpighian tubules of *Tenebrio*.

To our knowledge the mealworm *Tenebrio molitor* provides the first known example of antagonistic interactions between endogenous neuropeptides acting on Malpighian tubules and this study is the first to demonstrate the presence of K_{ATP} channels in an insect epithelium.

GENERAL INTRODUCTION

Insects, with their large surface area to volume ratio, need to carefully control the balance between water loss and uptake. This homeostasis is even more remarkable when one considers the variety of environments insects inhabit and their dietary intake, which ranges from very dry foods (e.g. *Tenebrio*) to large and infrequent blood meals (e.g. *Rhodnius).* The composition and volume of the haemolymph in insects is largely determined by the excretory system, which consists of the Malpighian tubules and the hindgut. Insect excretory systems work on the same broad principles as other renal organs. The formation of primary urine (or filtrate), which contains most haemolymph solutes, and the active secretion of some substances occurs in the Malpighian tubules, while the selective reabsorption of water, ions and essential metabolites occurs in the hindgut. Transport across these epithelia is controlled by diuretic and antidiuretic hormones.

Fluid secretion by Malpighian tubules

The mechanisms of fluid secretion by insect Malpighian tubules have been reviewed by several authors (Nicolson, 1993; Pannabecker, 1995; Beyenbach, 1995). Until recently, active transport of K^+ and/or Na^+ ions from the cell to the lumen was believed to be *via* a common cation pump (Maddrell, 1977). It has, however, become clear that a vacuolar-type proton pump is responsible for the apical transport of cations (Wieczorek, 1991). The V-ATPase pumps H^+ from the cytosol into the tubule lumen and establishes an electrochemical gradient for H^+, which drives an apical Na^+/nH^+ or K^+/nH^+ antiporter. The recycling of protons *via* this antiporter leads to secondary active extrusion of Na^+ or K^+ from the cell into the lumen (Pannabecker, 1995). Cl^- ions follow passively, either paracellularly or *via* a transcellular shunt, with water moving down the osmotic gradient (Dijkstra et al., 1994). Basolateral Na^+ or K^+ transport may occur passively *via* channels, by electroneutral $Na^+/K^+/2Cl$ or K^+/Cl^- cotransporters or by the Na^+/K^+-ATPase (O'Donnell and Maddrell, 1984; Leyssens et al., 1994).

Diuretic peptides

In general the diuretic peptides (DPs) which control fluid secretion by Malpighian tubules fall into three groups: the corticotropin-releasing factor (CRF)-related peptides, the kinins and the calcitonin-like peptides.

CRF-related peptides range from 30-47 amino acid residues in length (Baldwin et al., 2001) and show some structural relationship to the vertebrate CRF/urotensin/sauvagine family of peptides. Peptides of this family stimulate Malpighian tubule secretion by increasing the intracellular cyclic AMP concentration (Audsley et al., 1995). Some electrophysiological studies indicate that cyclic AMP increases cation (Na^+ and/or K^+) transport by increasing the conductance of the basolateral, cyclic AMP-sensitive Na^+ or K^+ channels (Beyenbach, 1995) or the basal $Na^+/K^+/2Cl^-$cotransporter (Hegarty et al., 1991; Ianowski and O'Donnell, 2001). In other studies there is evidence that cyclic AMP increases cation transport through the apical antiporter by stimulating the V-ATPase (O Donnell et al., 1998).

The insect kinins were first identified from the cockroach *Leucophaea maderae* (leucokinins) on the basis of their effect on the spontaneous contractile activity of the hindgut (Holman et al., 1991). In contrast to the CRF-related peptides, kinins are small (6-15 amino acid residues) and have a highly conserved C-terminal pentapeptide sequence. Kinins have no effect on cyclic AMP levels, but produce a very rapid increase in intracellular Ca^+ (Coast, 1998), to increase the anion permeability of Malpighian tubules. Studies with perfused tubules of *Aedes aegypti* indicate that kinins open a shunt pathway for Cl^-, changing the tubule from a "tight" to a "leaky" epithelium (Beyenbach, 1995). In *Drosophila melanogaster* tubules, kinins are thought to open a transcellular shunt for Cl^- through the stellate cells (O'Donnell et. al., 1996). There is evidence that the kinins and the CRF-related peptides act synergistically to control tubule secretion: when *Locusta*-DP and locustakinin were tested together on locust Malpighian tubules, the increase in fluid secretion was greater than the sum of their individual activities (Coast, 1995). In this case synergism between the two DPs involved an interaction of the cyclic AMP and the inositol triphosphate/Ca^{2+} signalling pathways. Synergism between hormones controlling secretion may have physiological advantages, such as the fact that lower quantities of hormones are needed to stimulate tubules quickly (Maddrell et al., 1993).

Very little is known about the calcitonin-like peptides, which to date have only been isolated from *Diploptera punctata* and *Drosophila melanogaster* (Furuya et al., 2000a; Coast et al., 2001). In *Drosophila* the homologue of the cockroach calcitonin-like peptide appears to activate the apical membrane V-ATPase to increase secretion rates.

Diuresis or clearance in beetles?

There has been confusion in the literature as to whether a diuretic hormone should be defined as a hormone that causes increased fluid secretion by the Malpighian tubules or diuresis from the whole insect. In insects that inhabit very dry environments, such as the tenebrionids *Onymacris plana* and *Tenebrio molitor,* there is no physiological need for rapid fluid secretion: however, brain and corpora cardiaca extracts from these insects have potent stimulatory effects on the secretion rate of their Malpighian tubules (Nicolson and Hanrahan, 1986; Nicolson, 1992). *In vivo* experiments indicate that, when fluid is secreted rapidly by *Onymacris* Malpighian tubules, it is directed to the midgut for recycling to the haemolymph (Nicolson, 1991). The effect of such recycling is that in these insects diuretic hormones, which should rather be termed clearance hormones, increase fluid secretion by the Malpighian tubules without affecting overall water balance.

Antidiuretic peptides

Previously it was assumed that declining fluid secretion by Malpighian tubules was the result of excretion or inactivation of the diuretic hormones, while antidiuretic hormones reduced excretion by stimulating hindgut reabsorption. There have been numerous studies on hindgut reabsorption but to date, the only well-defined peptide with antidiuretic effects on the hindgut is the ion transport peptide (ITP) of locusts. ITP has been shown to promote Cl^-, K^+, Na^+ and fluid reabsorption, and to inhibit H^+ secretion across locust ileum (Phillips and Audsley, 1995).

Antidiuretic factors which act directly on isolated Malpighian tubules have now been found in haemolymph and corpora cardiaca extracts of the cricket *Acheta domesticus* (Spring and Clark, 1990), whole body extracts of the mosquito *Aedes aegypti* (Petzel and Conlon, 1991) and head extracts of forest ants *Formica polyctena* (Laenen et al.,

2001), but the identity of the factors involved is unknown. Two antidiuretic peptides have recently been isolated from *T. molitor*, ADFa and ADFb, consisting of 13 and 14 amino acids respectively (Eigenheer et al., 2002; Eigenheer et al. in press)(Paper 3). These peptides inhibit fluid secretion by the Malpighian tubules *via* an increase in intracellular cyclic GMP. Quinlan et al. (1997) have shown that cyclic GMP also inhibits fluid secretion in *Rhodnius* tubules, but an increase in intracellular cyclic GMP of *D. melanogaster* tubules was shown to stimulate fluid secretion *via* the nitric oxide pathway (Dow et al., 1998).

Another peptide, the cardioacceleratory peptide 2b (CAP_{2b}), originally isolated as a myotropic peptide from the hawkmoth *Manduca sexta*, was shown to have an antidiuretic effect on the Malpighian tubules of *Rhodnius* (Quinlan et al., 1997), but stimulates fluid secretion in *D. melanogaster via* cyclic GMP (Davies et al., 1995; Terhzaz et al., 1999).

The physiological role of antidiuretic peptides with a direct inhibitory effect on the Malpighian tubules of insects is not clear and even less is known about the mechanisms that trigger the release of these peptides. In crickets, dehydration triggered the release of an antidiuretic factor from the corpora cardiaca (Spring et al., 1988). In blood feeders the release could also be triggered indirectly by a decline in haemolymph volume or $[Na^+]:[K^+]$ ratio as the blood meal is processed (Quinlan et al., 1997).

Aim of the present study

This study is a detailed investigation of the physiology and control of fluid secretion by Malpighian tubules of the beetle *Tenebrio molitor*. The availability of synthetic endogenous diuretic and antidiuretic factors that act directly on its Malpighian tubules makes *Tenebrio* an ideal model system (Furuya et al., 1995, 1998; Eigenheer et al., 2002; Eigenheer et al. in press).

To reach this goal widely differing experimental procedures were used.

Firstly, fluid secretion rates were measured by the use of a modified Ramsay assay. More detailed information about this bioassay is given in the Materials and Methods

section of Paper 1. Five to eight tubules were tested in each experiment, making this a fast and effective way of screening numerous stimulatory/inhibitory substances. Recently, O' Donnell and Spring, (2000) described various modes of hormonal control in insect Malpighian tubules. With the use of this assay, Paper 1 examines these modes of control for Malpighian tubules of *Tenebrio.*

Secondly, the localization of the endogenous peptides in the CNS of *Tenebrio* was determined by immunohistochemical procedures. The indirect peroxidase method was used for the screening of tissue sections, after which three-dimensional images were obtained by viewing whole mount immunofluorescent samples by confocal microscopy (for methods see Paper 2). Other investigators carried out the isolation and the characterization of diuretic and antidiuretic peptides as well as the conjugation and raising of the antisera. Paper 2 investigates the extensive distribution of Tenmo-DH_{37} in the CNS, corpora cardiaca and midgut of *Tenebrio* and the localization of the endogenous antidiuretic peptide Tenmo-ADFb is described in Paper 3.

Thirdly, electrophysiological techniques were applied to investigate mechanisms of ion transport across the epithelium. Transepithelial (V_{te}) and basolateral (V_{bl}) membrane potentials were measured using conventional microelectrodes and the apical membrane potential (V_{ap}) was determined indirectly by subtracting V_{te} from V_{bl}.

In Malpighian tubules of *Tenebrio* transepithelial K^+ transport appears to be dependent on the K^+ concentration of the surrounding haemolymph. Paper 4 investigates the effect of this regulatory mechanism on the basal and apical membrane and by the use of blockers examines the possible K^+ uptake mechanisms present in the basolateral membrane of *Tenebrio* tubule cells. The possibility of K^+ uptake *via* K_{ATP}-dependent K^+ channels (K_{ATP}) is investigated in Paper 5. This study is the first to provide evidence of (K_{ATP}) in an insect epithelium.

The final paper (Paper 6) investigates the possible modes of action of the endogenous diuretic peptide, Tenmo-DH_{37} and the antidiuretic peptide, Tenmo-ADFa, as well as their second messengers, cyclic AMP and GMP. In fluid secretion, an increase in Na^+ and/or K^+ uptake across the basolateral membrane must coincide with an increase in

apical extrusion of these cations into the tubule lumen. This study shows the effect of the endogenous peptides on the apical and basal transport mechanisms.

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Paper 1

Antagonistic control of fluid secretion by the Malpighian tubules of *Tenebrio molitor*: effects of diuretic and antidiuretic peptides and their second messengers

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Journal of Experimental Biology **205**, 493–501 (2002)

Contribution of co-authors other than supervisor(s)

R.A. Eigenheer isolated and synthesized the antidiuretic peptide Tenmo-ADFa. Prof. D.A. Schooley constructed the dose response graphs, and the isolation and synthesis of the antidiuretic peptide Tenmo-ADFa was done in his laboratory.

Note: **Paper 1** is written in the first person for thesis purposes.

Abstract

Fluid secretion by insect Malpighian tubules is controlled by haemolymph-borne factors. The mealworm *Tenebrio molitor* provides the first known example of antagonistic interactions between endogenous neuropeptides acting on Malpighian tubules. The two corticotropin-releasing-factor (CRF)-related diuretic peptides previously isolated from *Tenebrio molitor*, Tenmo-DH_{37} and Tenmo-DH_{47}, were found to stimulate *Tenebrio molitor* tubules *in vitro* in a dose-dependent manner with EC_{50} values of 0.12 nmol l^{-1} and 26 nmol l^{-1} respectively. However, no synergistic or additive effect was observed when these two peptides were tested simultaneously. I then investigated antagonism between second messengers: dose–response curves were constructed for stimulation of *Tenebrio molitor* tubules by cyclic AMP and their inhibition by cyclic GMP. When both cyclic nucleotides were included in the bathing Ringer, the stimulatory effect of cyclic AMP was neutralized by cyclic GMP. Similarly, the stimulatory effect of Tenmo-DH_{37} was reversed on addition of an antidiuretic peptide (Tenmo-ADF), which was recently isolated from *Tenebrio molitor* and acts *via* cyclic GMP.

The cardioacceleratory peptide CAP_{2b}, originally isolated from *Manduca sexta*, also increases intracellular cyclic GMP levels and inhibited fluid secretion by *Tenebrio molitor* tubules, with an EC_{50} value of 85 nmol l^{-1}. This inhibitory effect was reversed by Tenmo-DH_{37}. Endogenous diuretic and antidiuretic peptides, effective at low concentrations and acting *via* antagonistic second messengers, have the potential for fine control of secretion rates in the Malpighian tubules of *Tenebrio molitor*.

Keywords: diuretic peptide, antidiuretic peptide, cyclic AMP, cyclic GMP, antagonism, fluid secretion, Malpighian tubule, mealworm, *Tenebrio molitor*, Coleoptera.

Introduction

Fluid secretion by insect Malpighian tubules is stimulated by diuretic hormones. The need for diuretic hormones in blood feeders such as *Rhodnius prolixus* is obvious: however, diuretic activity is also present in the corpora cardiaca of two tenebrionid beetles inhabiting arid environments, the desert beetle *Onymacris plana* and the mealworm *Tenebrio molitor* (Nicolson and Hanrahan, 1986; Nicolson, 1992). *In vivo*

experiments indicate that the fluid secreted by stimulated Malpighian tubules of *Onymacris plana* is directed to the midgut for eventual recycling to the haemolymph (Nicolson, 1991). On the basis of these findings, Nicolson (1991) suggested that such diuretic hormones act as 'clearance hormones', because they elicit rapid clearance of metabolic waste from the haemolymph without loss of precious water. Insect diuretic hormones include serotonin (Barrett and Orchard, 1990; Maddrell et al., 1991) and diuretic peptides of at least three groups: the corticotropin-releasing-factor (CRF)-related peptides, the smaller kinins (Coast, 1996) and the more recently discovered calcitonin-related peptides (Furuya et al., 2000b; Coast et al., 2001). The 13 known CRF-related peptides (Baldwin et al., 2001) share various degrees of homology with vertebrate CRF and appear to act through the second messenger cyclic AMP to increase the rate of cation transport (Audsley et al., 1993; Beyenbach, 1995; O'Donnell et al., 1996). They are the only family of diuretic peptides for which unequivocal evidence is available that they serve a hormonal function (Patel et al., 1995). Insect kinins make up the second major family of diuretic peptides. Kinins were initially isolated on the basis of their myotropic activity on the hindgut of the cockroach *Leucophaea maderae* and range in length from six to 13 amino acid residues (Coast, 1996). They have a highly conserved COOH-terminal pentapeptide sequence and appear to act through an increase in intracellular Ca^{2+} concentration, which increases the anion permeability of the Malpighian tubules (O'Donnell et al., 1998). Only two calcitonin-like peptides have been fully identified to date; their signal transduction pathway appears to be complex, largely involving elevation of intracellular Ca^{2+} concentrations in *Locusta migratoria* (Furuya et al., 2000b) but of cyclic AMP concentrations in *Drosophila melanogaster* (Coast et al., 2001).

Since the first CRF-related diuretic peptide was isolated from *Manduca sexta* (Kataoka et al., 1989), peptides belonging to this family have also been isolated from Orthoptera, Dictyoptera, Coleoptera and Diptera (for a review, see Coast, 1996). In the sphinx moths *Manduca sexta* and *Hyles lineata* (Kataoka et al., 1989; Blackburn et al., 1991; Furuya et al., 2000a) and in the beetle *Tenebrio molitor*, two CRF-related diuretic peptides have been isolated from one species. Furuya et al. (1995, 1998) used whole head extracts of *Tenebrio molitor* pupae to isolate and characterize two peptides of 37 and 47 amino acid residues (Tenmo-DH_{37} and Tenmo-DH_{47} respectively). Both stimulate fluid secretion in *Tenebrio molitor* tubules *via* the

production of intracellular cyclic AMP, but Tenmo-DH_{47} is 600 times less potent in this assay than Tenmo-DH_{37} and lacks the C-terminal asparagine (free acid) 'extension' of Tenmo-DH_{37}, which suggests that another receptor may exist in a different tissue for Tenmo-DH_{47} (Furuya et al., 1998). The significance of two structurally related diuretic peptides in an insect species, acting *via* the same second messenger, is not yet clear (Furuya et al., 1998, 2000a).

It has commonly been assumed that, when antidiuretic factors are present in insects, they reduce fluid loss by stimulating hindgut reabsorption (Spring, 1990). Antidiuretic factors that act directly on isolated Malpighian tubules have been demonstrated in haemolymph and corpora cardiaca extracts of the cricket *Acheta domesticus* (Spring et al., 1988), whole-body extracts of the mosquito *Aedes aegypti* (Petzel and Conlon, 1991), head and abdominal extracts of the forest ant *Formica polyctena* (De Decker et al., 1994; Laenen et al., 2001) and head extracts of the Colorado potato beetle *Leptinotarsa decemlineata* (Lavigne et al., 2001), but the identity of the factors involved is unknown. Eigenheer et al. (2002) have isolated from *Tenebrio molitor* an antidiuretic peptide (Tenmo-ADF), consisting of 14 amino acid residues, which inhibits fluid secretion by the Malpighian tubules *via* cyclic GMP as a second messenger. An increase in intracellular cyclic GMP concentration has the same effect in *Rhodnius prolixus* tubules (inhibits fluid secretion) (Quinlan et al., 1997), but in *Drosophila melanogaster* tubules cyclic GMP was shown to stimulate fluid secretion *via* the nitric oxide pathway (Dow et al., 1994). Opposing effects on tubule secretion are also caused by the cardioacceleratory peptide 2b (CAP_{2b}), originally isolated as a myotropic peptide from *Manduca sexta*: this has an antidiuretic effect on the Malpighian tubules of *Rhodnius prolixus* (Quinlan et al., 1997), but stimulates fluid secretion in *Drosophila melanogaster* by raising intracellular cyclic GMP levels (Davies et al., 1995).

The availability of synthetic endogenous peptides makes the mealworm *Tenebrio molitor* an ideal but non-traditional model for examining the complexities of control of fluid balance by Malpighian tubules. In this study, I investigate the effects on fluid secretion of the diuretic and antidiuretic peptides isolated from *Tenebrio molitor* and of their second messengers cyclic AMP and cyclic GMP. I investigate possible synergism between the two diuretic peptides, Tenmo-DH_{37} and Tenmo-DH_{47}, and

possible antagonism between the peptides and their second-messenger signalling pathways (O'Donnell and Spring, 2000). I also examine the effects of CAP_{2b} on Malpighian tubules of *Tenebrio molitor*, and compare the secretory responses of larval and adult tubules. This is the first study to investigate the interactions between endogenous diuretic and antidiuretic peptides, isolated from the same insect, that act directly on the Malpighian tubules.

Materials and methods

Insects

Tenebrio molitor L. larvae and adults used in this study were maintained in dry bran cultures at room temperature (20–23 °C). Their diet was supplemented weekly with apple or potato as a source of moisture. Care was taken to select mealworms of similar size for the secretion experiments.

Ringer's solution

The Ringer's solution used for isolated tubules of *Tenebrio molitor* (Nicolson, 1992) contained (in mmol l^{-1}): NaCl, 90; KCl; 50; MgCl2, 5; CaCl2, 2; NaHCO3, 6; NaH2PO4, 4; glycine, 10; proline, 10; serine, 10; histidine, 10; glutamine, 10; and glucose, 50. The pH was adjusted to 7.0 with NaOH.

Fluid secretion assays

Both larval and adult beetles have three pairs of large Malpighian tubules with conspicuous brown pigment. The six tubules vary in length according to their positions (dorsal, lateral or ventral pairs). Nicolson (1992) showed that there are no positional or regional differences in the secretion rates of larval tubules. The free portions of the tubules were dissected under Ringer's solution by securing the larva with two pins, opening the cuticle on the dorsal side, gently pulling the gut from the body and dissecting all six tubules free from the fat body, severing each one before it entered the rectal complex. It was not necessary to measure tubule length as each tubule served as its own control. All experiments utilized larval tubules unless stated otherwise. Tubules from adult beetles were isolated as described by Nicolson and Hanrahan (1986) for the tenebrionid beetle *Onymacris plana*.

Malpighian tubules were set up as *in vitro* preparations at room temperature (20–23 °C) using the technique first described by Ramsay (1954) with a few modifications. Two tubules were isolated into each 50 μl drop of Ringer under water-saturated liquid paraffin in a Petri dish with a Sylgard-covered base. Both ends of each tubule were pulled out of the bathing fluid and wrapped around minuten pins, where they continued to secrete fluid (usually at one end only) which collected as discrete droplets in the liquid paraffin. The tubules were allowed to equilibrate for 20 min before three control readings were taken at 15 min intervals. Secreted drops were removed from the tubule with a fine glass pipette, and their diameter was measured with a calibrated eyepiece graticule as they rested on the Sylgard-covered base of the dish. The volume, and therefore the rate of secretion, was determined assuming the droplets to be spherical. After the control period, the Ringer was replaced with Ringer containing the test substance. The degree of stimulation or inhibition was expressed as a percentage of the last control rate reading. Dose–response curves for cyclic AMP, cyclic GMP and CAP_{2b} were constructed using the secretion rate measured 45 min after the addition of the test substance. Dose-response curves for the two diuretic peptides were constructed by first measuring secretion rates in the presence of the peptide, then adding fresh Ringer containing 0.1 mmol l^{-1} 8-bromo-cyclic AMP to obtain maximum rates.

Drugs and peptides

Cyclic AMP and cyclic GMP (sodium salts) and 8-bromocyclic AMP were purchased from Sigma. Tenmo-DH_{37} and Tenmo-DH_{47} were from batches synthesised and purified as described previously (Furuya et al., 1995, 1998). Tenmo-ADF was also from a batch synthesised recently (Eigenheer et al., 2002). CAP_{2b} was synthesised using Na-9-fluorenylmethoxycarbonyl (FMOC) chemistry with an Applied Biosystems 431A synthesiser using 0.1 mmol of 4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamidonorleucyl-MBHA (Rink amide MBHA) resin. The native peptide has a pyroglutamic acid residue on the N terminus; we synthesized it with a Gln residue at this position, which requires a subsequent cyclisation reaction to pyroglutamate in basic solution. After cleavage from the resin, 35 mg of the crude peptide (synthetic [Gln^1]CAP_{2b}) was dissolved in water and then brought to 0.1 mol l^{-1} triethylamine. This basic solution was allowed to react for 12 h at room temperature and then loaded onto a 22 mm Adsorbosphere 300XL C18 (300A) column and eluted

with a gradient of 0% to 60 % ethanol over 60 min with 0.1 % aqueous trifluoroacetic acid maintained throughout. The second of two major peaks contained the cyclised CAP_{2b}; the purity and identity were confirmed by electrospray ionization (ESI) mass spectrometry analysis with a Finnigan MAT SSQ instrument with ESI interface. Bovine serum albumin (0.05%) was included in the Ringer with all peptides.

Statistical analyses

Results are expressed as means ± standard error (S.E.M.). Statistical differences were calculated using paired or unpaired Student's *t*-tests. A difference was considered significant if $P<0.05$. Dose–response curves were fitted by non-linear regression analysis using Prism 3.0.

Results

Tenmo-DH$_{37}$ and Tenmo-DH$_{47}$: potency and possible synergism

Both diuretic peptides isolated from *Tenebrio molitor*, Tenmo-DH_{37} and Tenmo-DH_{47}, were potent stimulants of fluid secretion by isolated Malpighian tubules, with half-maximal response (EC_{50}) values in the nanomolar range (Fig. 1). The stimulation due to the diuretic peptide is expressed as a percentage of the response obtained with 8-bromo-cyclic AMP. Tenmo-DH_{37} was more potent, with an EC_{50} value of 0.12 nmol l^{-1} compared with 26 nmol l^{-1} for Tenmo-DH_{47}.

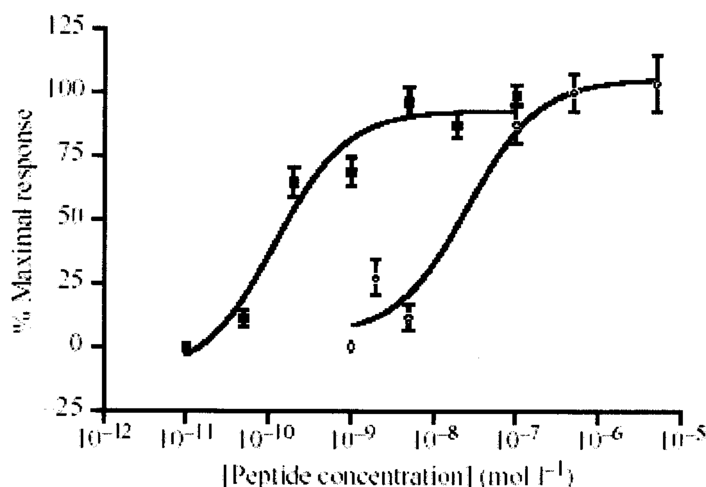

Fig. 1. Dose–response curves for the effect of *Tenebrio molitor* diuretic peptides Tenmo-DH_{37} (filled squares) and Tenmo-DH_{47} (open circles) on fluid secretion rates. Results are expressed as a percentage of the maximal response obtained in the presence of 8-bromo-cyclic AMP (0.1 mmol l^{-1}). Data points are the mean of 6–12 determinations for Tenmo-DH_{37} and 6–7 determinations for Tenmo-DH_{47}; vertical lines represent ± 1 S.E.M. The EC_{50} values are 0.12 nmol l^{-1} for Tenmo-DH_{37} and 26 nmol l^{-1} for Tenmo-DH_{47} (r^2 values for the curve fits were 0.95 and 0.96, respectively).

I then investigated whether these two diuretic peptides, acting through adenylyl cyclase, cooperate synergistically. Both peptides, when tested individually at approximately 1.5 times their respective EC_{50} value, increased tubule secretion by approximately 150 % (Fig. 2). When they were tested together, no further increase in secretion rate was observed. It seems, therefore, that these two peptides do not function additively or synergistically.

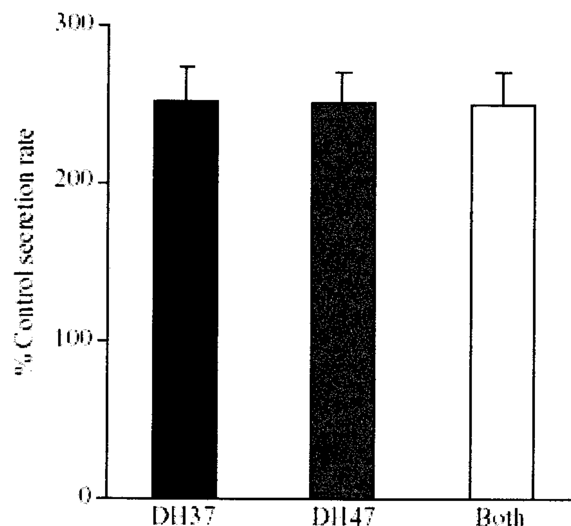

Fig. 2. Lack of synergism between Tenmo-DH_{37} (DH_{37}) and Tenmo-DH_{47} (DH_{47}). Diuretic peptides were tested at 0.2 nmol l^{-1} and 40 nmol l^{-1}, approximately 1.5 times their respective EC_{50} values, after which the Ringer's solution was changed to one containing both peptides (at these same concentrations). No change in secretion rates was observed. Data are presented as means + 1 S.E.M. (N=8).

Stimulation by cyclic AMP

The dose–response curve for *Tenebrio molitor* tubules stimulated by cyclic AMP is shown in Fig. 3. Maximum stimulation, 335±61 % of control rates, was produced by a cyclic AMP concentration of 0.5 mmol l^{-1} (N=7–12). The secretion rate declined at concentrations above 0.5 mmol l^{-1}, although this decline was not significant ($0.05<P<0.2$). Excluding the two highest cyclic AMP concentrations from the curve-fitting procedure gave an EC_{50} of 350 mmol l^{-1}. The speeds of the tubule response to cyclic AMP and Tenmo-DH_{37} were compared using concentrations resulting in maximal stimulation (100 nmol l^{-1} Tenmo-DH_{37} and 1 mmol l^{-1} cyclic AMP) (Fig. 4). Identical maximum rates of secretion were achieved by 15 min after addition of both stimulants.

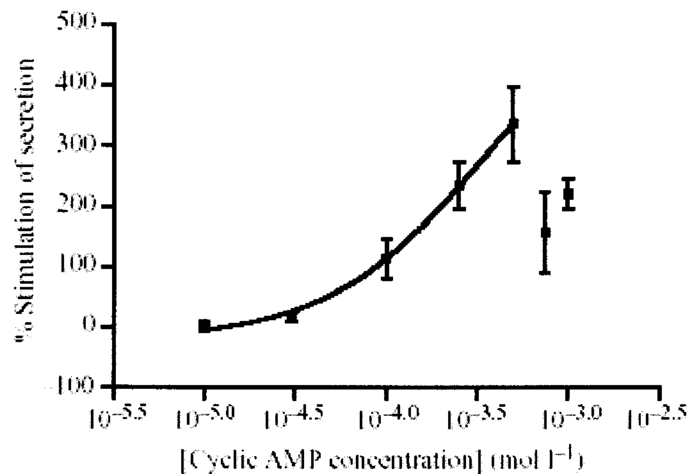

Fig. 3. Dose–response curve for the effect of cyclic AMP on fluid secretion by *Tenebrio molitor* Malpighian tubules. Data are presented as means ± S.E.M. of 7–12 tubules. The EC50 was 350 mmol l^{-1} (r^2=0.999 for the curve fit), using 0.5 mmol l^{-1} cyclic AMP as the highest concentration. Note that data for the two highest cyclic AMP concentrations tested (0.75 and 1 mmol l^{-1}) are shown in the graph, but were excluded from the non-linear regression analysis because they indicate some desensitization at high levels, and this cannot be accommodated by the algorithm used.

Inhibition by cyclic GMP

Cyclic GMP inhibits secretion by *Tenebrio molitor* tubules in a dose-dependent manner. At a concentration of 0.5 mmol l^{-1} cyclic GMP, fluid secretion was maximally inhibited by 50±6% (N=8; Fig. 5). The EC_{50} was 490 µmol l^{-1} for concentrations up to 0.5 mmol l^{-1} cyclic GMP. Zero inhibition does not occur, even at very low cyclic GMP concentrations, because of the decline in basal rates of secretion with time (e.g. Fig. 4). Only 0.5 mmol l^{-1} cyclic GMP gave a significant increase in inhibition when compared with the lowest cyclic GMP concentration used (t-tests, P<0.05). Fluid secretion rates however, were significant inhibited from doses higher than 0.1 mmol l^{-}.

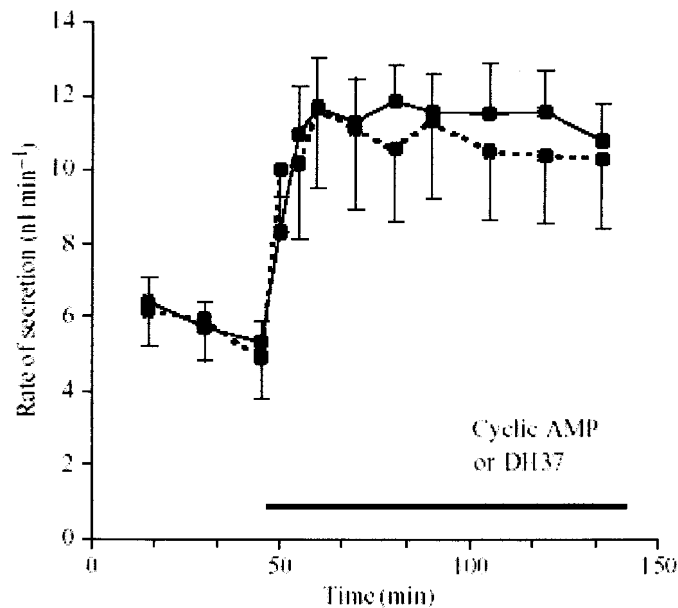

Fig. 4. Comparison of secretory response to cyclic AMP and Tenmo-DH_{37} (DH_{37}). No significant differences in the response time or maximum rates of secretion were observed when Malpighian tubules of *Tenebrio molitor* were stimulated with 1 mmol l^{-1} cyclic AMP (broken line) or 100 nmol l^{-1} Tenmo-DH_{37} (solid line). Each point shows the mean ± S.E.M. for 8–12 tubules. The horizontal filled bar indicates the period during which tubules were exposed to either cyclic AMP or Tenmo-DH_{37}.

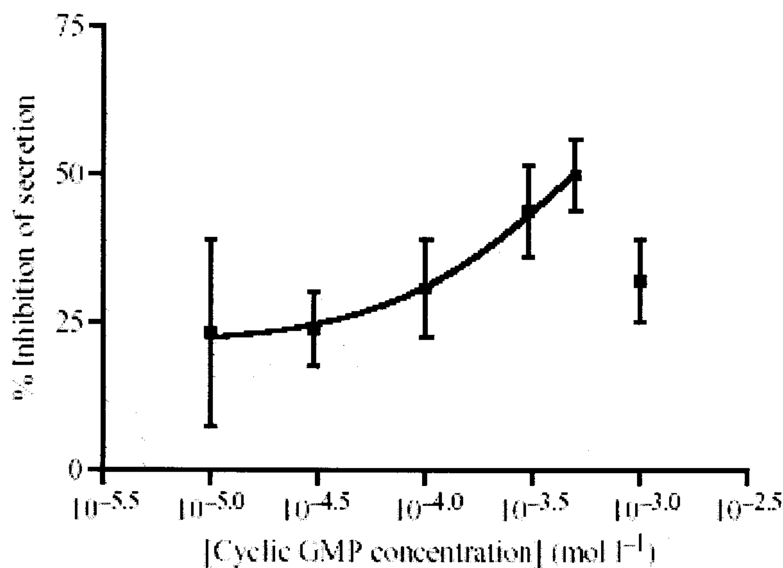

Fig. 5. Dose–response curve for the effect of cyclic GMP on fluid secretion. Data are presented as means ± 1 S.E.M., N=5–11. The EC_{50} value determined was 490 mmol l^{-1} (r^2=0.999, 95 % confidence intervals 99.7 mmol l^{-1} to 2.4 mmol l^{-1}). Note that data for the highest cyclic GMP concentration have been excluded from the non-linear regression analysis because they indicate some desensitization at high levels, and this cannot be accommodated by the algorithm used.

Antagonistic effects of peptides and their second messengers

Antagonism between the second messengers cyclic AMP and cyclic GMP in isolated tubule preparations is illustrated by Fig. 6. Cyclic GMP at 1 mmol l^{-1}, added to tubules already stimulated by 0.25 mmol l^{-1} cyclic AMP, effectively reduced secretion rates to the previously measured baseline level. Thus, in this particular experiment, a cyclic GMP concentration four times higher than the cyclic AMP concentration was necessary to neutralize the stimulatory effect of cyclic AMP.

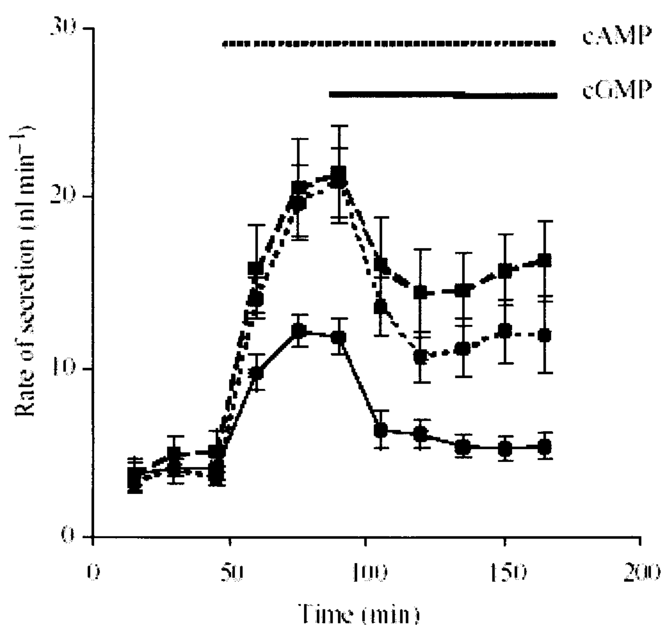

Fig. 6. Antagonistic effect of cyclic GMP on Malpighian tubules of *Tenebrio molitor* already stimulated with cyclic AMP. Each point shows the mean ± S.E.M. for 8–10 tubules. Responses are shown for 0.5 mmol l^{-1} cyclic AMP in combination with 0.5 mmol l^{-1} cyclic GMP (broken line), 0.5 mmol l^{-1} cyclic AMP with 1 mmol l^{-1} cyclic GMP (dashed line) and 0.25 mmol l^{-1} cyclic AMP with 1 mmol l^{-1} cyclic GMP (solid line). The horizontal dotted bar indicates the period during which tubules were exposed to cyclic AMP, and the horizontal filled bar indicates the period of exposure to cyclic GMP.

Fig. 7 shows the antagonistic effect of cyclic GMP on responses to cyclic AMP (both at concentrations of 0.5 mmol l^{-1}) and Tenmo-ADF on Tenmo-DH_{37} (both at concentrations of 100 nmol l^{-1}). Concentrations found to elicit maximal responses (inhibition or stimulation) were used. Secretion rates were measured after 45 min in control Ringer, 45 min after addition of fresh Ringer containing either Tenmo-DH_{37} or cyclic AMP, and finally 45 min after replacement with fresh Ringer containing

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both stimulant and inhibitor. In both experiments, the time course of stimulation and inhibition and the magnitude of the response were similar. In both experiments, secretion rates were significantly increased by the added stimulants (paired *t*-tests, $P<0.001$), but inclusion of the respective inhibitors returned the secretion rates to values not significantly different from control levels.

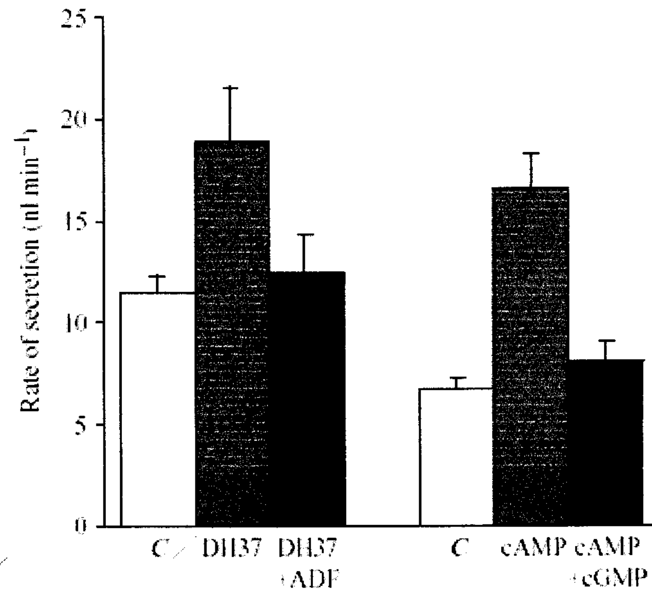

Fig. 7. Antagonism between *Tenebrio molitor* peptides controlling Malpighian tubule secretion and between their second messengers. Effect of adding Tenmo-ADF (ADF) to tubules already stimulated with Tenmo-DH_{37} (DH_{37}) (both at concentrations of 100 nmol l^{-1}) compared with the effect of adding cyclic GMP to tubules already stimulated with cyclic AMP (both at concentrations of 0.5 mmol l^{-1}). Secretion rates were measured after 45 min of each treatment. Values are means + 1 S.E.M. for eight tubules (peptides) or seven tubules (second messengers). *C*, control rates.

CAP_{2b} has an antidiuretic effect on Tenebrio molitor tubules

I investigated the effect of CAP_{2b} on the tubules of *Tenebrio molitor* by means of secretion assays and found that the peptide decreased the rate of fluid secretion in a dose dependent manner (Fig. 8), with 80±2 % inhibition of fluid secretion obtained at a concentration of 1 mmol l^{-1} CAP_{2b}. Again, zero inhibition was not obtained because of declining rates of secretion by tubules *in vitro*.

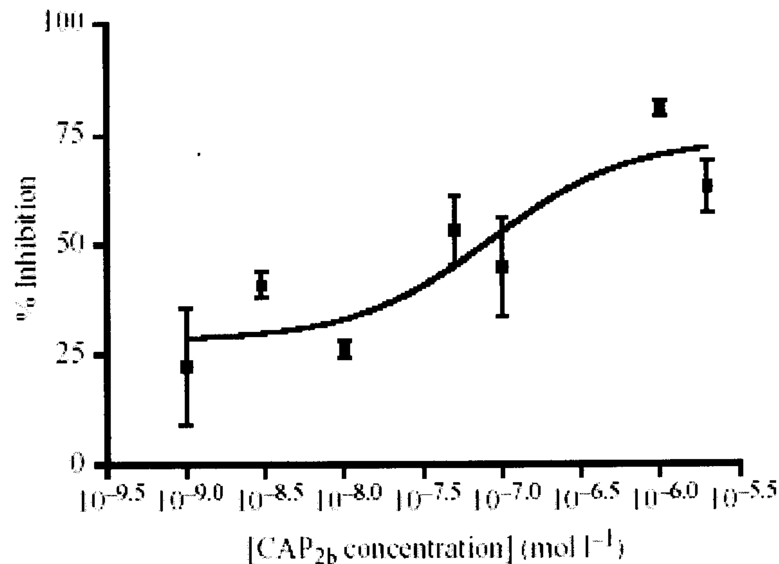

Fig. 8. Dose–response curve for the effect of CAP_{2b} on fluid secretion by *Tenebrio molitor* tubules. Data are presented as means ±1 S.E.M. for 8–9 tubules. The EC_{50} value determined was 85 nmol l^{-1} (r^2=0.79, 95 % confidence intervals 4.3 nmol l^{-1} to 1.7 mmol l^{-1}).

Fig. 9 illustrates the antagonism between Tenmo-DH_{37} and CAP_{2b} in tubules of T*enebrio molitor*, with inhibition preceding stimulation. Treatment with 1 mmol l^{-1} CAP_{2b} caused the secretion rate to drop sharply. When the Ringer was replaced with Ringer containing both 1 mmol l^{-1} CAP_{2b} and 10 nmol l^{-1} Tenmo-DH_{37}, the fluid secretion rate increased, but more slowly than during stimulation by Tenmo-DH_{37} alone in previous experiments (e.g. Fig. 4). There was a further increase in fluid secretion rate after removal of CAP_{2b} from the medium, significant after 30 min (paired *t*-test, $P<0.025$).

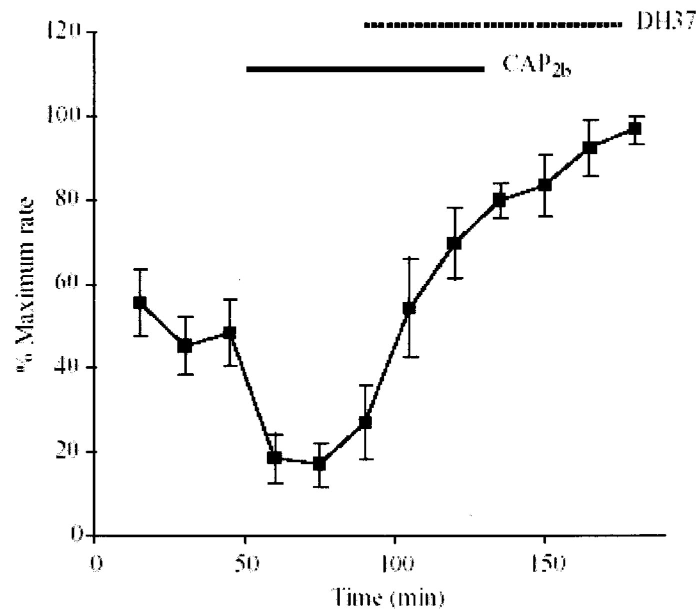

Fig. 9. Antagonism between CAP_{2b} and Tenmo-DH_{37} (DH_{37}). Inhibition of fluid secretion by 1 mmol l^{-1} CAP_{2b} and subsequent slow stimulation by Tenmo-DH_{37} (10 nmol l^{-1}). Data are presented as means ±1 S.E.M. for five tubules. The horizontal filled bar indicates the period of exposure to CAP_{2b}, and the horizontal dotted bar indicates the period of exposure to Tenmo-DH_{37}.

Comparison of larval and adult tubules

The tubules of *Tenebrio molitor* larvae and adults have the same appearance and arrangement in relation to the gut. I compared the effect of 100 nmol l^{-1} Tenmo-DH_{37} and 1 mmol l^{-1} cyclic AMP on the secretion rates of larval and adult tubules (Fig. 10) and found no difference in magnitude of stimulation of adult or larval tubules treated with either stimulant (P>0.05). There was also no difference in the time to reach the maximal rate of secretion (not shown).

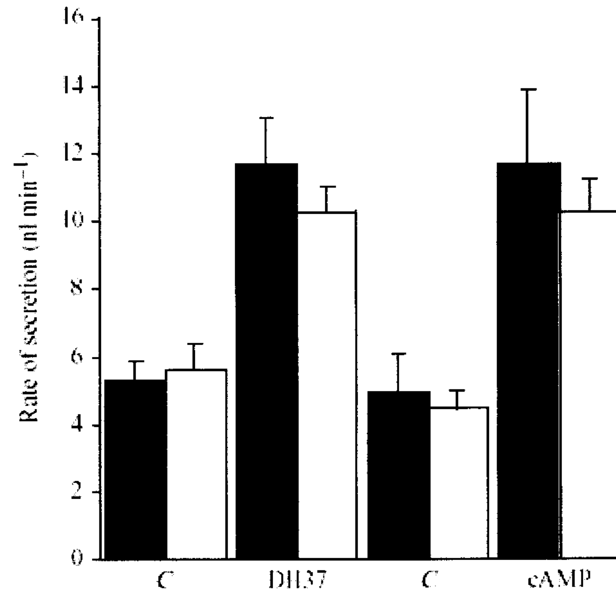

Fig. 10. Effects of stimulants on secretion rates of *Tenebrio molitor* larval (filled columns) and adult (open columns) Malpighian tubules. Tubules were stimulated with 100 nmol l^{-1} Tenmo-DH_{37} (DH_{37}) and 1 mmol l^{-1} cyclic AMP. Data are presented as means + 1 S.E.M. (*N*=9–10). *C*, control rates.

Discussion

The complexity of hormonal control of insect Malpighian tubules becomes increasingly apparent. Recently, O'Donnell and Spring (2000) reviewed various modes of control, including synergism between diuretic factors, involving one or more second-messenger systems, and a single example of antagonism between controlling factors (serotonin and CAP_{2b}) and their second messengers in *Rhodnius prolixus* (Quinlan et al., 1997). The availability of synthetic endogenous peptides, both diuretic and antidiuretic, has enabled us to examine the complexities of control of *Tenebrio molitor* Malpighian tubules.

Stimulation of fluid secretion (Tenmo-DH_{37}, Tenmo-DH_{47} and cyclic AMP)

The blood-sucking bug *Rhodnius prolixus* has long been a model for studies of Malpighian tubule fluid and ion secretion because of the dramatic diuresis that follows after an infrequent but massive blood meal. In this insect, two different stimulants (serotonin and a CRF-related peptide), both acting *via* cyclic AMP as a second messenger, cause acceleration of fluid secretion by isolated tubules (Maddrell et al., 1993; Te Brugge et al., 1999).

A synergistic secretory response to two different peptides, one a CRF-related peptide and the other a kinin, has been demonstrated in Malpighian tubules of *Locusta migratoria* and *Musca domestica* (Coast, 1995; Iaboni et al., 1998). These peptides utilize different second messengers, and synergism appears to involve an interaction between the cyclic AMP and inositol triphosphate/Ca^{2+} signalling systems (O'Donnell and Spring, 2000). The advantage of such synergism is that the dose–response curve is effectively steepened and the tubules are stimulated more quickly at lower peptide concentrations (Coast, 1995). Synergistic effects have also been observed in the cockroach *Diploptera punctata* for a calcitonin-like peptide, Dippu-DH_{31}, and the CRF-like peptide Dippu-DH_{46} isolated from this species (Furuya et al., 2000b). The former peptide is believed to act *via* elevation of intracellular Ca^{2+} concentration, while the latter elevates cyclic AMP concentration. Curiously, in *Locusta migratoria*, Dippu-DH_{31} acts synergistically with locustakinin, an elevator of intracellular Ca^{2+} concentration, as well as with the locust CRF-like peptide Locmi-DH (Furuya et al., 2000b).

In *Tenebrio molitor*, two CRF-related peptides, Tenmo-DH_{37} and Tenmo-DH_{47}, both acting *via* cyclic AMP, cause diuresis in isolated tubule preparations. As in the desert tenebrionid *Onymacris plana*, in which the extract of 0.1 pairs of corpora cardiaca in 100 ml of Ringer's solution caused an increase in fluid secretion rate up to 100 nl min^{-1} (Nicolson and Hanrahan, 1986), mealworm tubules are highly sensitive to low doses of the diuretic peptides, which is curious considering the dryness of their diet and environment. Physiologically, it is not clear why these xeric insects have such potent diuretic hormones, but evidence exists that these hormones act instead as clearance hormones, recirculating the tubule fluid to moisten the midgut contents and filtering the haemolymph of metabolic waste (Nicolson, 1991, 1992). The effect of such recycling is that diuretic hormones increase fluid secretion by the Malpighian tubules without affecting the overall water balance of the insect.

The two diuretic peptides characterised from *Tenebrio molitor* do not act additively or synergistically (Fig. 2). Although an additive response would be expected when both peptides are at submaximal concentrations, the response may be minimal on the steep part of the dose–response curve. A similar lack of additivity was found by Furuya et al. (2000b) when testing the CRF-like peptides Locmi-DH and Dippu-DH_{46} on locust

tubules. The possibility exists that Tenmo-DH_{47}, which is approximately 200 times less potent in stimulating fluid secretion and 600 times less potent in stimulating cyclic AMP production by the tubule cells (Furuya et al., 1998), has a different function. As far as synergism between CRF-related peptides and kinins is concerned, no kinins have yet been isolated from *Tenebrio molitor*, and the kinins of other insects (leucokinins II and VII, achetakinin and muscakinin) induce no response in isolated tubules of *Tenebrio molitor* (U. I. M. Wiehart, unpublished data).

Inhibition of fluid secretion (Tenmo-ADF, CAP_{2b} and cyclic GMP)

Exogenous cyclic GMP inhibits secretion by Malpighian tubules of *Tenebrio molitor* at concentrations between 0.1 mmol l^{-1} and 1 mmol l^{-1} (Fig. 5). In contrast, tubules of *Drosophila melanogaster* and the black field cricket *Teleogryllus oceanicus* are stimulated by both cyclic AMP and cyclic GMP (Davies et al., 1995; O'Donnell et al., 1996; Xu and Marshall, 2000), while perfused tubules of *Aedes aegypti* show no electrical response to dibutyryl cyclic GMP (Clark et al., 1998). The latter study demonstrated dose-dependent effects of *Culex salinarius* CRF-related peptide on tubules of another mosquito, *Aedes aegypti*. The effects of CAP_{2b} and cyclic GMP on the fluid secretion rates and the transepithelial potential of *Drosophila melanogaster* tubules are also concentration-dependent (Davies et al., 1995). The opposing effects of cyclic AMP and cyclic GMP on secretion rates of *Tenebrio molitor* tubules were reduced at concentrations around 1 mmol l^{-1} compared with the response at somewhat lower concentrations (Figs 3, 5). This may be due to desensitization, and the same effect is evident in the secretory response of tubules to Tenmo-ADF (Eigenheer et al., 2002). In *Drosophila melanogaster* tubules, fluid secretion is stimulated by low doses of cyclic GMP or CAP_{2b}, but the effect is concentration-dependent; at high concentrations, the initial stimulation is followed by a decline in fluid secretion rate to control levels (Davies et al., 1995).

Cyclic GMP elicits an antagonistic effect in *Tenebrio molitor* tubules previously stimulated with cyclic AMP. The relative concentration of cyclic GMP needed to neutralize the cyclic AMP effectively varied in different experiments (cf. Figs 6 and 7). Mealworm tubules with low control rates of secretion showed the greatest stimulation with extracts of corpora cardiaca (Nicolson, 1992) and similarly I found that, when control rates were lower, the tubules showed a more dramatic response to

cyclic AMP, and a higher relative concentration of cyclic GMP was necessary to antagonise this response.

In *Rhodnius prolixus*, cyclic GMP is thought to antagonize cyclic AMP by activating cyclic AMP phosphodiesterases and thus speeding the degradation of cyclic AMP (O'Donnell and Spring, 2000). This mode of action may also hold for the tubules of *Tenebrio molitor*, in which cyclic AMP phosphodiesterase inhibitors block the effects of antidiuretic peptides on cyclic GMP production and fluid secretion (R. A. Eigenheer, S. W. Nicolson and D. A. Schooley, unpublished data).

In *Drosophila melanogaster* tubules, CAP_{2b} elevates cyclic GMP levels *via* an increase in nitric oxide (NO) concentration (Davies et al., 1995); this occurs through modulation of an endogenous NO synthase, which leads to activation of soluble guanylyl cyclase and increases intracellular levels of cyclic GMP. In contrast, the NO donor sodium nitroprusside does not affect fluid secretion by tubules of *Rhodnius prolixus*, which suggests that a different type of guanylyl cyclase (membrane associated) is involved in cyclic GMP synthesis in this insect (Quinlan et al., 1997). Although the effect of NO donors was not investigated on fluid secretion, Eigenheer et al. (2002) have shown that these donors have no effect on cyclic GMP levels in *Tenebrio molitor* tubules.

Hormones with an antidiuretic effect on Malpighian tubules are little known in insects, and the physiological role of these factors remains ambiguous (Laenen et al., 2001). The antidiuretic peptide isolated from *Tenebrio molitor* heads, Tenmo-ADF, strongly inhibits fluid secretion by isolated tubule preparations and effectively antagonizes the stimulatory response of Tenmo-DH_{37} (Fig. 7). This extremely potent antidiuretic inhibits fluid secretion in the femtomole range by increasing cyclic GMP production in the Malpighian tubules on a dose-dependent basis (Eigenheer et al., 2002). It seems appropriate for a xeric insect such as *Tenebrio molitor* to have such a potent antidiuretic factor. However, as in the desert beetle *Onymacris plana*, homogenates of the corpora cardiaca stimulate secretion in *Tenebrio molitor* tubules, suggesting that diuretic factors predominate in these crude extracts (Nicolson and Hanrahan, 1986; Nicolson, 1992). Similarly, although extracts of the metathoracic ganglion of *Rhodnius prolixus* were found to elevate intracellular cyclic GMP levels

in Malpighian tubules, the diuretic factors present predominate in fluid secretion assays (Quinlan et al., 1997). It is not known whether the antidiuretic peptide Tenmo-ADF promotes reabsorption of fluid by the mealworm cryptonephric complex. To date, there are only two well-defined peptides that stimulate hindgut reabsorption in insects. One of these is ion-transport peptide (ITP) isolated from *Schistocerca gregaria*, which has no stimulatory or inhibitory action on fluid secretion by locust Malpighian tubule preparations (Coast et al., 1999). The other is *Manduca sexta* diuretic hormone (Manse-DH), which increases fluid uptake from the rectal sac of M*anduca sexta* larvae in addition to stimulating the free portions of the Malpighian tubules (Audsley et al., 1993); this combination of diuretic and antidiuretic actions in the same insect would result in recycling of fluid by the excretory system. Immunocytological localization of Tenmo-ADF may give us some indication of function in the hindgut of *Tenebrio molitor*.

The data presented here, in the first study of the physiological actions of diuretic and antidiuretic peptides isolated from the same insect species, illustrate the potentially intricate control of fluid secretion by *Tenebrio molitor* Malpighian tubules. Several questions for further research on diuresis and antidiuresis are raised by this work. First, do the diuretic and antidiuretic peptides isolated from *Tenebrio molitor* mediate diuresis or antidiuresis *in vivo* and what triggers the release of these hormones? Second, what are the cellular effects of the second messengers and how do they modulate specific ion transporters of the tubule cells to cause changes in secretion rates? Finally, in what tissues are these peptides localized and how does the distribution of the diuretic and antidiuretic peptides compare?

Financial support was provided by NSF Grant IBN 9602148, the Nevada Agricultural Experiment Station, the University of Cape Town and the South African National Research Foundation. I thank Kathleen M. Schegg for synthesis of CAP_{2b}.

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Paper 2

Immunocytochemical localization of a diuretic hormone of the beetle *Tenebrio molitor*, Tenmo-DH_{37}, in nervous system and midgut

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Cell Tissue Research **308**: 421-429 (2002)

Abstract

Although the mealworm *Tenebrio molitor* inhabits very dry environments, it has at least two diuretic peptides, which increase fluid secretion by the free portions of the Malpighian tubules. Unlike other insect CRF-related peptides isolated to date, these are non-amidated peptides. The immunocytochemical localization of Tenmo-DH_{37} was investigated using antisera raised against this hormone. Immunoreactive neurosecretory cells were found in the brain and abdominal ganglia with immunoreactive processes projecting to the peripheral nervous system. Intense staining of the neurohaemal release site, the corpora cardiaca, was observed. In addition, neurosecretory cells immunoreactive to Tenmo-DH_{37} were found in the posterior midgut and a network of immunoreactive nerve processes extended over the surface of the midgut. Tenmo-DH_{37} is widely distributed and its staining pattern resembles that found for other, amidated CRF-related diuretic peptides.

Keywords: Neurosecretory cells - CRF-related peptides - Malpighian tubules – Diuresis - *Tenebrio molitor* (Coleoptera, Insecta)

Introduction

Insect diuretic peptides belong to three families, of which the two best characterized are the CRF-related diuretic peptides and the myokinins (Coast 1996). The 13 known CRF-related peptides (Table 1) share various degrees of homology with vertebrate corticotropin-releasing factor (CRF), and utilize cyclic AMP as second messenger to increase cation transport (Beyenbach 1995; O'Donnell et al. 1996). The smaller kinins were initially isolated on the basis of their myotropic activity on the hindgut of *Leucophaea maderae* and range in size from 6-15 amino acids (Coast 1996). They have a highly conserved COOH-terminal pentapeptide sequence and appear to act through an increase of intracellular Ca^{2+} to increase anion permeability of the Malpighian tubules (O'Donnell et al. 1998). Recently calcitonin-like peptides have been identified in *Diploptera punctata* (Furuya et al. 2000a) and *Drosophila melanogaster* (Coast et al. 2001). Little is known about the mode of action of these diuretic peptides and their signal

Contribution of co-authors other than supervisor(s)

P. Torfs raised the antiserum against Tenmo-DH_{37} used for the immunocytochemical studies.

A. Van Lommel helped to obtain publication standard images with the confocal microscope.

All the experimental work was done in Prof. Schoofs laboratory.

Note: **Paper 2** is written in the first person for thesis purposes.

transduction pathways appear complex, involving largely elevation of intracellular Ca^{2+} in *Locusta migratoria* but cyclic AMP in *Drosophila melanogaster* (Coast et al. 2001).

The ability of a diuretic factor to stimulate primary urine production *in vitro* does not necessarily indicate its function as a diuretic hormone *in vivo*. For example, achetokinins have been shown to have diuretic activity, but also cause cockroach hindgut contraction, as well as lipid mobilization and inhibition of protein synthesis in crickets and locusts (Patel et al. 1994). For a diuretic peptide to be classified as a hormone, several criteria must be met (Coast 1996). The peptide must be synthesized in neurosecretory cells and transported to neurohaemal structures from where it is released into the circulation, and such a release should occur under appropriate physiological stimulus conditions. The resultant increase in haemolymph titre should produce an effect in the target tissue (Malpighian tubules), which can be mimicked by synthetic peptide and blocked by receptor antagonists. The CRF-related peptide which comes closest to meeting these criteria is *Locusta*-DP, which immunocytochemistry has shown to be localized in the CNS, corpora cardiaca and perivisceral organs (Patel et al. 1994). Moreover, *Locusta*-DP is colocalized with a leucokinin-like peptide, locustakinin, both peptides acting additively to promote Malpighian tubule fluid secretion (Thompson et al. 1995). CRF-related peptides have also been found localized in nerve tissue of *Manduca* (Veenstra and Hagedorn 1991), *Musca* (Iaboni et al. 1998) and *Rhodnius* (Te Brugge et al. 1999).

Recently two CRF-related peptides were isolated and characterized from *Tenebrio molitor*, containing 37 and 47 amino acid residues, respectively (Furuya et al. 1995, 1998). In contrast to all other CRF-related peptides, Tenmo-DH_{37} and Tenmo-DH_{47} are not C-terminally amidated (Table 1). Tenmo-DH_{37} is a very potent diuretic factor with an EC_{50} value for fluid secretion by *Tenebrio* Malpighian tubules of 0.12 nM (Wiehart et al. 2002). Like the desert tenebrionid beetle *Onymacris plana*, *Tenebrio molitor*, which lives on dry bran, is a most unlikely insect to possess a potent diuretic factor. Previous studies on *Onymacris* showed that the high *in vitro* rates of fluid secretion by the Malpighian tubules in response to extracts of corpora cardiaca are not associated with diuresis *in vivo* (Nicolson 1991). Nicolson therefore suggested that the term "clearance hormones" is

more appropriate for these diuretic peptides, especially when isolated from insects that inhabit arid environments.

In this paper I brought Tenmo-DH_{37} one step closer to being a hormone by describing the extensive distribution of DH_{37} immunoreactivity in the CNS, corpora cardiaca and midgut of *Tenebrio*. I accomplished this by the development of an antiserum which is specific for Tenmo-DH_{37} and does not cross-react with the amidated CRF-related peptides from other insects. I further show that the increase in tubule secretion elicited by Tenmo-DH_{37} can be reversed by the antiserum.

Peptide	Species	Sequence		Reference
Tenmo-DH_{37}	*Tenebrio molitor*	SPTISITAPIDVLRKTWEQERARKQMVKNREFLNSLN.................... -	OH	Furuya et al. 1995
Tenmo-DH_{47}	*Tenebrio molitor*	AGALGESGASLSIVNSLDVLRNRLLEIARKKAKEGANRNRQILLSL... -	OH	Furuya et al. 1998
Manse-DH	*Manduca sexta*	RMPSLSIDLPMSVLRQKLSLEKERKVHALRAAANRNFLNDI............ -	NH_2	Kataoka et al. 1989
Manse-DPII	*Manduca sexta*	SFSVNPAVDILOHRYMEKVAQNNRNFLNRV............................. -	NH_2	Blackburn et al. 1991
Hylli-DH_{41}	*Hyles lineata*	RMPSLSIDLPMSVLRQKLSLEKERKVQALRAAANRNFLNDI........... -	NH_2	Furuya et al. 2000b
Hylli-DH_{30}	*Hyles lineata*	SFSVNPAVEILQHRYMEKVAQNNRNFLNRV.............................. -	NH_2	Furuya et al. 2000b
Mud-DP	*Musca domestica*	NKPSLSIVNPLDVLRORLLLEIARROMKENTRQVELNRAILKNV...... -	NH_2	Clottens et al. 1994
Culsa-DH	*Culex salinarius*	TKPSLSIVNPLDVLRQRIILEMARRQMRENTRQVERNKAILREI........ -	NH_2	Clark et al. 1998
Peram-DP	*Periplaneta americana*	TGSGPSLSIVNPLDVLRQRLLLEIARRRMRQSQDQIQANREILQTI....	NH_2	Kay et al. 1992
Zoone-DH	*Zootermopsis nevadensis*	TGAVPSLSIVNPLDVLRQRLLLEIARRRMRQSQDQIQANREMLQTI.. -	NH_2	Baldwin et al. 2001
Dippu-DH_{46}	*Diploptera punctata*	TGTGPSLSIVNPLDVLRQRLLLEIARRRMRQTQNMIQANRDFLESI... -	NH_2	Furuya et al. 2000a
Locmi-DH	*Locusta migratoria*	MGMGPSLSIVNPMDVLRQRLLLEIARRRLRDAEEQIKANKDFLQQI. -	NH_2	Kay et al. 1991a
Achdo-DP	*Acheta domesticus*	TGAQSLSIVAPLDVLRQRLMNELNRRRMRELQGSRIQQNRQLLTS.. -	NH_2	Kay et al. 1991b

Table 1 The primary structures of the CRF-like diuretic peptides that have been isolated to date. Both diuretic peptides isolated from *Tenebrio molitor* are unique in having a more hydrophilic, non-amidated COOH terminus.

Materials and methods

Animals

Tenebrio molitor was kept under crowded conditions at room temperature (20-23°C) and fed on a diet of bran and apple. All experiments were performed on mealworms of similar size. Adult locusts aged 2-3 weeks from the colony maintained at Catholic University of Leuven were used for a cross-species study.

Antiserum development

Synthetic Tenmo-DH_{37} was a gift from D A Schooley (Department of Biochemistry, University of Nevada, Reno). One mg of Tenmo-DH_{37} was coupled to bovine thyroglobulin using glutaraldehyde. An equal volume of 2% glutaraldehyde in phosphate-buffered saline (PBS) was added to a solution containing bovine thyroglobulin and Tenmo-DH_{37} in a molar ratio of 10:1 (peptide:carrier) in PBS. After 1 h of incubation at room temperature with constant agitation, glycine was added to a final concentration of 200 mM, and the reaction was incubated with stirring for another hour. Excess reagent was separated from the hapten-carrier complex by dialysis. Two rabbits were immunized over a period of three months. Principles of laboratory animal use and specific national laws were followed. The complex was dissolved in distilled water and emulsified with an equal amount of Freund's complete adjuvant and injected subcutaneously into New Zealand white rabbits. After initial immunization, four booster injections were given at 3-week intervals. The antisera were collected one week after the last injection.

A dot immunobinding assay (Salzet et al. 1997) was used to characterize the antisera. One µl aliquots of Tenmo-DH_{37} were spotted onto a nitrocellulose membrane (0.45 µm pore size) in a dilution series ranging from 100 ng up to 1 µg/dot. Membranes were baked (30 min, 110°C), blocked with skimmed milk in 50 mM TRIS-buffered saline (TBS) to reduce background staining (1 h gentle agitation; room temperature), and then incubated overnight at 4°C with the Tenmo-DH_{37} antiserum (diluted 1:1000 in TBS). Membranes were washed several times and incubated with peroxidase-conjugated goat anti-rabbit IgG for 2 h. Immunoreactive spots were visualized with hydrogen peroxide as an enzyme substrate and 3-3'-diaminobenzidine as chromogenic reagent.

Preparation of tissue

The CNS (brain, suboesophageal ganglion, ventral nerve cord), corpora cardiaca and midgut of *Tenebrio* larvae as well as brains and corpora cardiaca from locusts were dissected under physiological saline and transferred to Bouin-Hollande's (10%) sublimated fixative. After 18-24 h fixation the tissue was thoroughly rinsed with distilled water (12 h), dehydrated in an ethanol series (70-100% for 2 × 1h each), cleared in xylene and embedded in Paraplast. Alternating sections (4 μm) were cut with an LKB Historange microtome, using glass knives.

Immunocytochemical methods

Sections were stained using the indirect immunoperoxidase peroxidase method (IIPO). Following a preincubation period of 45 min with pre-immune goat serum (PIG) to avoid non-specific binding, the tissue sections were incubated overnight in a moist chamber with the primary antibody. For this purpose the antiserum was used at a dilution of 1:500 in 0.01M TBS, pH 7.6. After a 2×5 min rinse with TBS, the slides were incubated in a 1:200 dilution of anti-rabbit peroxidase conjugated secondary antibody. The excess secondary antibody was removed after 1 h with 0.01M TBS, pH 7.6. The peroxidase-conjugated antibodies were revealed by a staining reaction using hydrogen peroxide (0.01%) as an enzyme substrate and 3-3'-diaminobenzidine (125 μg/ml) as chromogenic reagent. A staining time of 2 min was considered to be optimum.

Whole-mount immunofluorescence

Nervous tissue and midguts were dissected from larvae under physiological saline, fixed in 2% paraformaldehyde in 10 mM PBS, pH 7.2 for 18-24 h and subsequently washed in PBS for 4-5 h. To enhance antibody penetration and reduce non-specific binding, tissues were incubated overnight in 4% Triton X-100, containing 2% normal porcine serum (NPS) and 2% bovine serum albumin (BSA). The primary DH_{37}-antiserum was applied in a dilution of 1:500. Prior to incubation for 72 h on a flatbed shaker at 4 °C, it was preabsorbed in 0.4% Triton X-100, containing 2% NPS and 2% BSA. Tissues were washed in cold PBS for 4-5 h prior to incubation in the secondary antibody for 24 h at 4 °C. The secondary antibody was FITC (fluorescein isothiocynanate)-conjugated swine

anti-rabbit immunoglobulins (DAKO) diluted 20x with PBS. Tissues were washed in PBS, mounted in 90% glycerol containing the anti-fading reagent, para-phenylenediamine (PPD) (0.1%) and viewed by confocal microscopy (Zeiss LSM 410).

Serum specificity

Specificity of the antiserum was determined by application of the pre-immune rabbit antiserum taken from the animal that produced the primary antiserum. To avoid false positive results the application of this homologous non-immune serum is recommended (Tips et al. 1991). The specificity of the immunocytochemical staining was determined by preabsorbing 1ml of Tenmo-DH_{37} antiserum with 100 μg and 250 μg of synthetic Tenmo-DH_{37} at 4 °C overnight. The antiserum was also preabsorbed with Tenmo-DH_{37} (100 ng/ml) in the secretion assay.

Isolated tubule preparations

Malpighian tubules from larval *Tenebrio* were isolated as described by Wiehart et al. (2002). The Ringer solution used for dissecting tubules and the secretion assay contained (in mM): NaCl 90, KCl 50, $MgCl_2$ 5, $CaCl_2$ 2, $NaHCO_3$ 6, NaH_2PO_4 4, glycine 10, proline 10, serine 10, histidine 10, glutamine 10, and glucose 50. The pH was adjusted to 7.0 with NaOH. Two tubules were isolated into each 50 μl drop of Ringer under water-saturated liquid paraffin in a Sylgard-lined Petri dish. The ends of each tubule were pulled out of the bathing fluid and wrapped around Minuten pins, where they continued to secrete; the urine collected as discrete droplets in the liquid paraffin. Secreted drops were removed with a fine glass pipette and their diameters measured with a calibrated eyepiece graticule. The volume, and therefore the rate of secretion, was determined assuming the droplets to be spherical. The tubules were allowed to equilibrate for 20 min before three control readings were made at 15 min intervals. The Ringer was then replaced with Ringer containing Tenmo-DH_{37} (0.2 nM) and measurements were taken over a 60 min period. For the final 60 min the tubules were exposed to Tenmo-DH_{37} and antiserum (1:500 dilution). Rates of secretion were expressed as a percentage of the third control rate reading.

Results

Antiserum development and specificity

Characterization by dot immunobinding assay revealed that the developed antisera used in a dilution of 1:1000 recognized Tenmo-DH_{37} with a detection limit of 10 pg/dot. Omitting or replacing the primary antiserum with non-immune rabbit antiserum produced no staining. Preabsorption of the antiserum with 250 µg of synthetic Tenmo-DH_{37} resulted in complete abolition of staining in the CNS and midgut of *Tenebrio*. In addition, the antisera did not stain the neuroendocrine cells or other processes in the locust brain which have been shown to be immunoreactive to Locmi-DH (see Patel et al. 1994).

I tested the effect of the antiserum on secretion rate by *Tenebrio* Malpighian tubules, previously stimulated with 0.2 nM Tenmo-DH_{37}. The secretion rate of tubules stimulated with Tenmo-DH_{37} increased from 5.2 ± 0.94 to 14.8 ± 2.80 nl/min after 60 min (Fig. 1). When the Ringer solution was replaced with Ringer containing 0.2 nM Tenmo-DH_{37} preabsorbed with a 1:500 dilution of the antiserum for 30 min, the secretion rate dropped back to 4.3 ± 0.79 nl/min after 60 min, which was not significantly different from the control rate levels ($P = 0.056$). The antiserum at a 1:500 dilution had no significant effect on the secretion rate of control tubules (Fig 1).

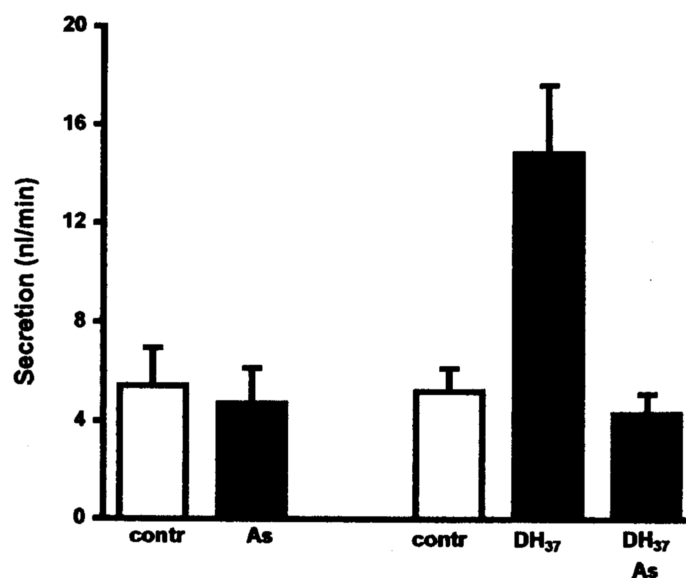

Fig 1 Preabsorption with antiserum raised against the peptide completely reversed the stimulatory response of *Tenebrio* tubules to Tenmo-DH_{37}. The stimulatory effect of 0.2 nM Tenmo DH_{37} was completely reversed by the addition of 0.2 nM Tenmo-DH_{37} previously preabsorbed with the antiserum. A 1:500 dilution of the antiserum had no effect on control secretion rates (*left*). Each data point represents the mean $\pm$ 1 S.E.M. (*vertical lines*) of 8 tubules.

Immunocytochemistry

Tenmo-DH_{37} immunoreactive material was found distributed in the protocerebrum of the brain, the corpora cardiaca, the abdominal ganglia and nerves, and the midgut of *Tenebrio molitor*. Two neuroendocrine systems, in particular, displayed very strong staining: 1) cells of the pars intercerebralis that project to the storage lobe of the corpora cardiaca, and 2) abdominal neurons that project to the perivisceral organs.

Tenmo-DH_{37} immunoreactivity in the brain and corpora cardiaca

A group of 24-30 cells in the brain showed positive staining, with 10-12 cells staining very intensely. These neurosecretory cells, approximately 15-20 μm in diameter, were found on the ventral midline of the protocerebrum in the pars intercerebralis (Fig. 2A, B). The cell bodies projected axons posteriorly to produce intertwined dendritic processes that continued on either side of the midline (Fig. 2A, C). No extensive arborization of the projections from the cell bodies was seen and no immunoreactive cells were found in the deuto- and tritocerebrum.

The nervi corporis cardiaci I (NCCI) originate from neurosecretory cells in the pars intercerebralis; they include axons which innervate the corpora cardiaca and terminate in this organ. Some dentritic processes from the neurosecretory cells continued to the neuropil of the brain. The complete neuronal pathway is visible in Fig. 2C: immunoreactive axons from the two groups of neurosecretory cells cross before leaving the brain and continue in the NCC I to the contralateral corpus cardiacum. Here the immunoreactive cells form varicosities (Fig. 2D), termed blebby processes in locust corpora cardiaca by Patel et al. (1994). Staining of the entire corpora cardiaca was very dense due to the numerous immunoreactive endings found there.

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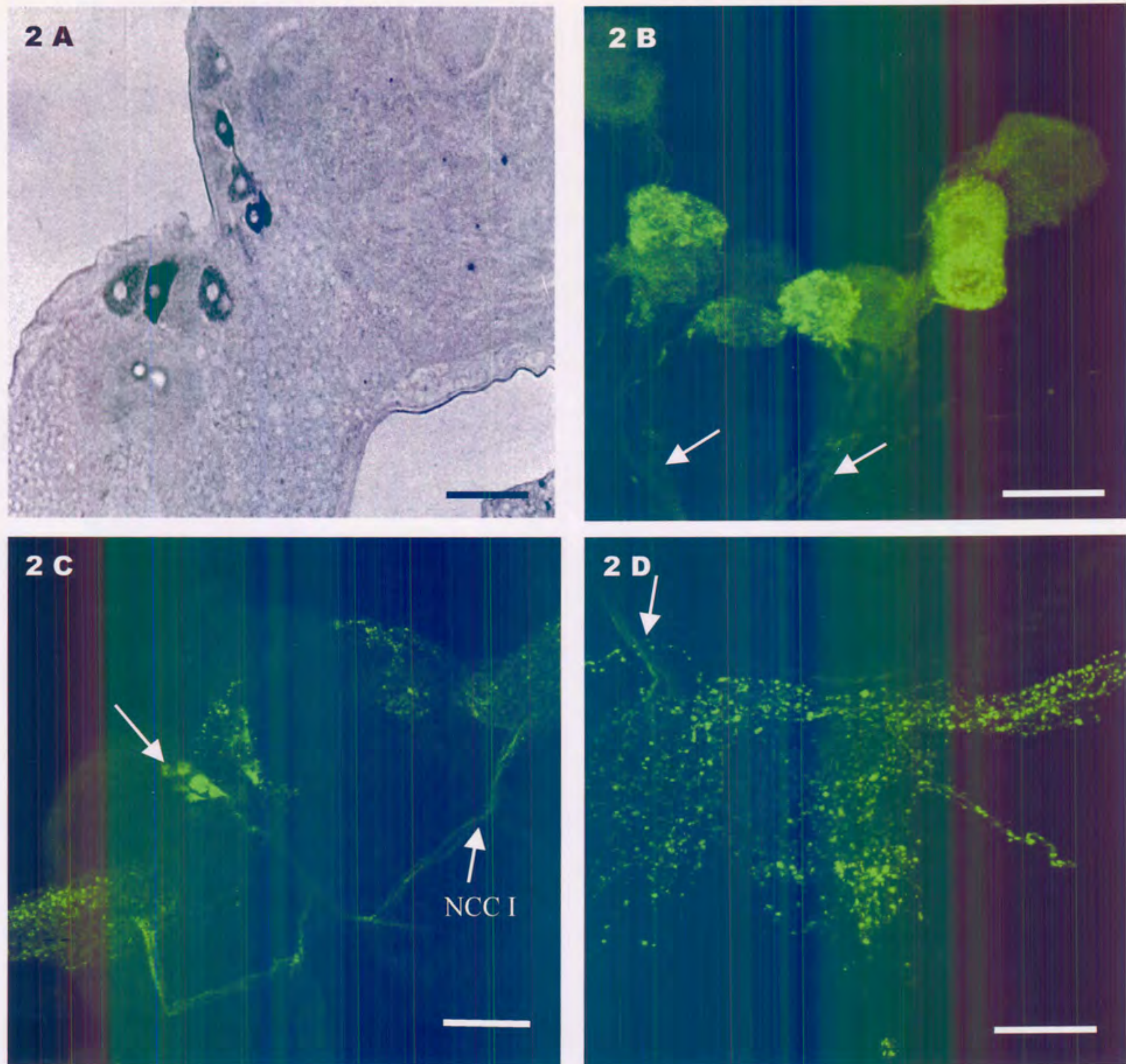

Fig 2A-D Tenmo-DH_{37} immunoreactivity in brain and corpora cardiaca. Anterior is to the top, left (except in **D**). **A** Transverse section of the brain stained with anti-Tenmo-DH_{37} antiserum showing immunoreactive cell bodies located on the ventral midline in the pars intercerebralis. Some cell bodies stained stronger than others (IIPO method). Scale bar: 20 µm **B** Immunoreactive cell bodies of the pars intercerebralis demonstrated by whole mount immunofluorescence. Cell bodies on either side of the midline project axons posteriorly (arrows). Scale bar: 25 µm **C** Whole mount immunofluorescence of the brain of *T. molitor* demonstrates the complete neuronal pathway. Tenmo-DH_{37}-neurosecretory cells (long arrow) in the pars intercerebralis with their axons running in the direction of the contralateral corpus cardiacum (displaced laterally) *via* the NCC I. Some dendritic processes continue to the neuropil of the brain (short arrow). Scale bar: 50 µm **D** Neurosecretory processes from the cell bodies in the pars intercerebralis terminate in the corpora cardiaca, the major neurosecretory organ of the brain, as varicose extensions. The NCC I innervate this organ dorsally (arrow). Scale bar: 25 µm

DH_{37} immunoreactivity in the ventral nerve cord

The ventral nerve cord of the *Tenebrio* larva consists of a suboesophageal-, three thoracic-, and eight abdominal ganglia. No immunoreactive staining was found in the suboesophageal ganglion or in the three thoracic ganglia in any preparation. Two clearly staining cell bodies, 20-25 μm in diameter, were found situated posterio-laterally on each side of all the abdominal ganglia (A1-A7) (Fig 3A, B). A weaker staining third cell was apparent in some of the ganglia (Fig 3C). Each cell body projects an axon directed to the midline, which continues anteriorly and posteriorly (Fig 3A, C), and an efferent axon that exits the ganglion at the ventral nerve. The arrangement of cells with their ipsilateral projection pattern is obviously bilaterally and segmentally homologous. These efferent axons enter intense staining processes on either side of the ventral nerve. The staining of these processes resembles that of a neurohaemal release site (Fig 3A, B). These abdominal Tenmo-DH_{37} efferents were not traced to their peripheral targets in the body. The arrangement of the cell bodies in the last abdominal ganglion (A8) is different to that of the preceding ganglia. A group of 4 or 5 large cells in the center of the ganglion showed strong immunoreactivity (Fig 3D). These cells project processes posteriorly, which enter into a dense plexus of varicose Tenmo-DH_{37}-immunoreactive material. Two efferent axons exit this meshwork in two of the terminal nerves (Fig 3E), to possibly become part of the peripheral nervous system.

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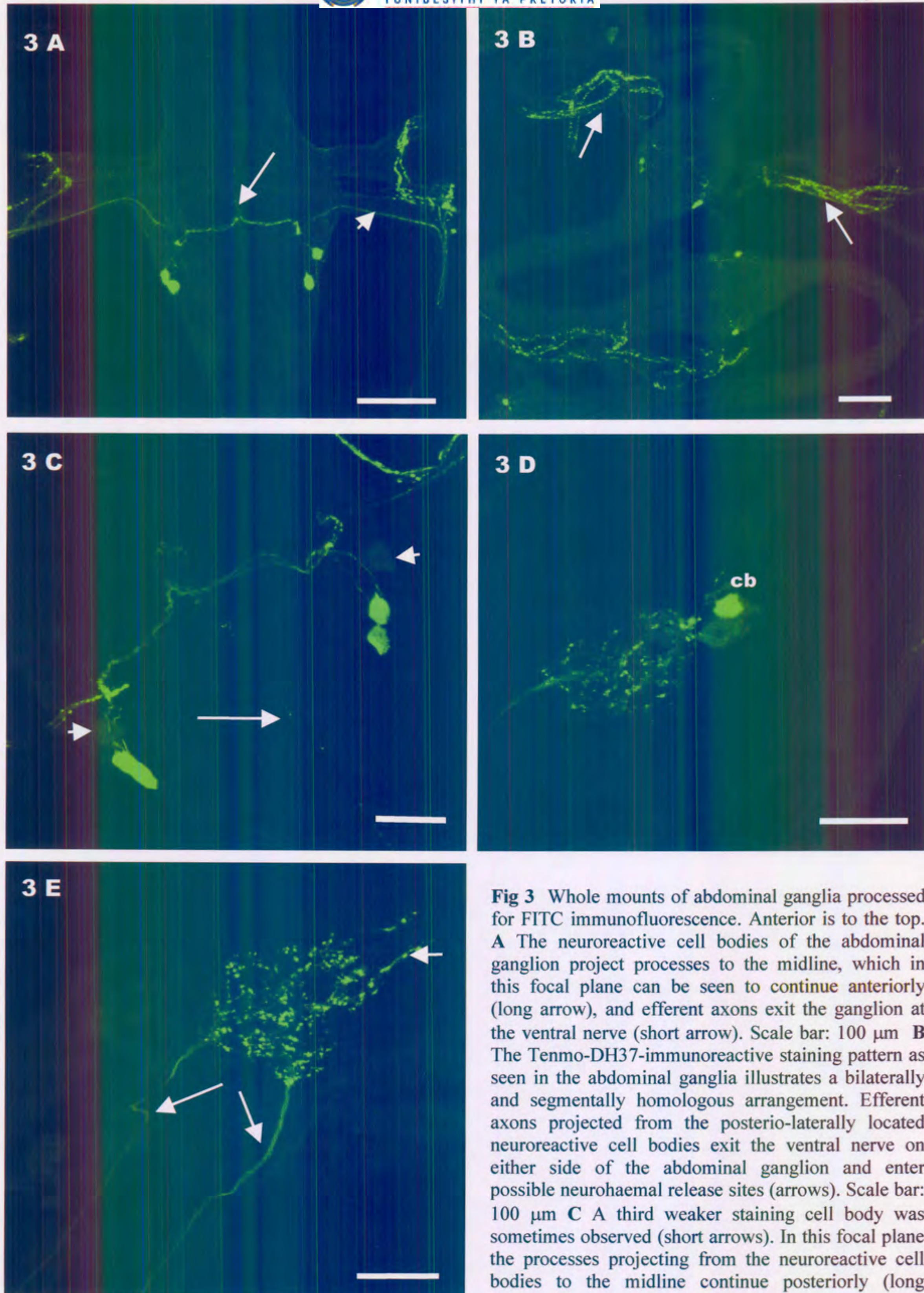

Fig 3 Whole mounts of abdominal ganglia processed for FITC immunofluorescence. Anterior is to the top. **A** The neuroreactive cell bodies of the abdominal ganglion project processes to the midline, which in this focal plane can be seen to continue anteriorly (long arrow), and efferent axons exit the ganglion at the ventral nerve (short arrow). Scale bar: 100 μm **B** The Tenmo-DH37-immunoreactive staining pattern as seen in the abdominal ganglia illustrates a bilaterally and segmentally homologous arrangement. Efferent axons projected from the posterio-laterally located neuroreactive cell bodies exit the ventral nerve on either side of the abdominal ganglion and enter possible neurohaemal release sites (arrows). Scale bar: 100 μm **C** A third weaker staining cell body was sometimes observed (short arrows). In this focal plane the processes projecting from the neuroreactive cell bodies to the midline continue posteriorly (long arrow). Scale bar: 50 μm **D,E** The immunoreactive cells in the terminal ganglion (**cb**) are large and centrally located with posteriorly projecting processes that enter a dense plexus (short arrow). Two efferent axons exit the plexus via two of the terminal nerves (long arrows). Scale bars: 100 μm

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DH_{37} immunoreactivity in the midgut

Immunoreactive staining in the midgut was consistent in all the preparations studied. Numerous strongly staining immunoreactive cells were found distributed in the posterior part of the midgut (Fig. 4A). These triangular shaped cells with their slender ends facing the gut lumen have a central nucleus and intensely staining cytoplasm (Fig. 4B). The abundance of these endocrine-like cells declined towards the anterior part of the midgut. Fine nerve processes could be seen running over the surface of the entire midgut (Fig. 4C).

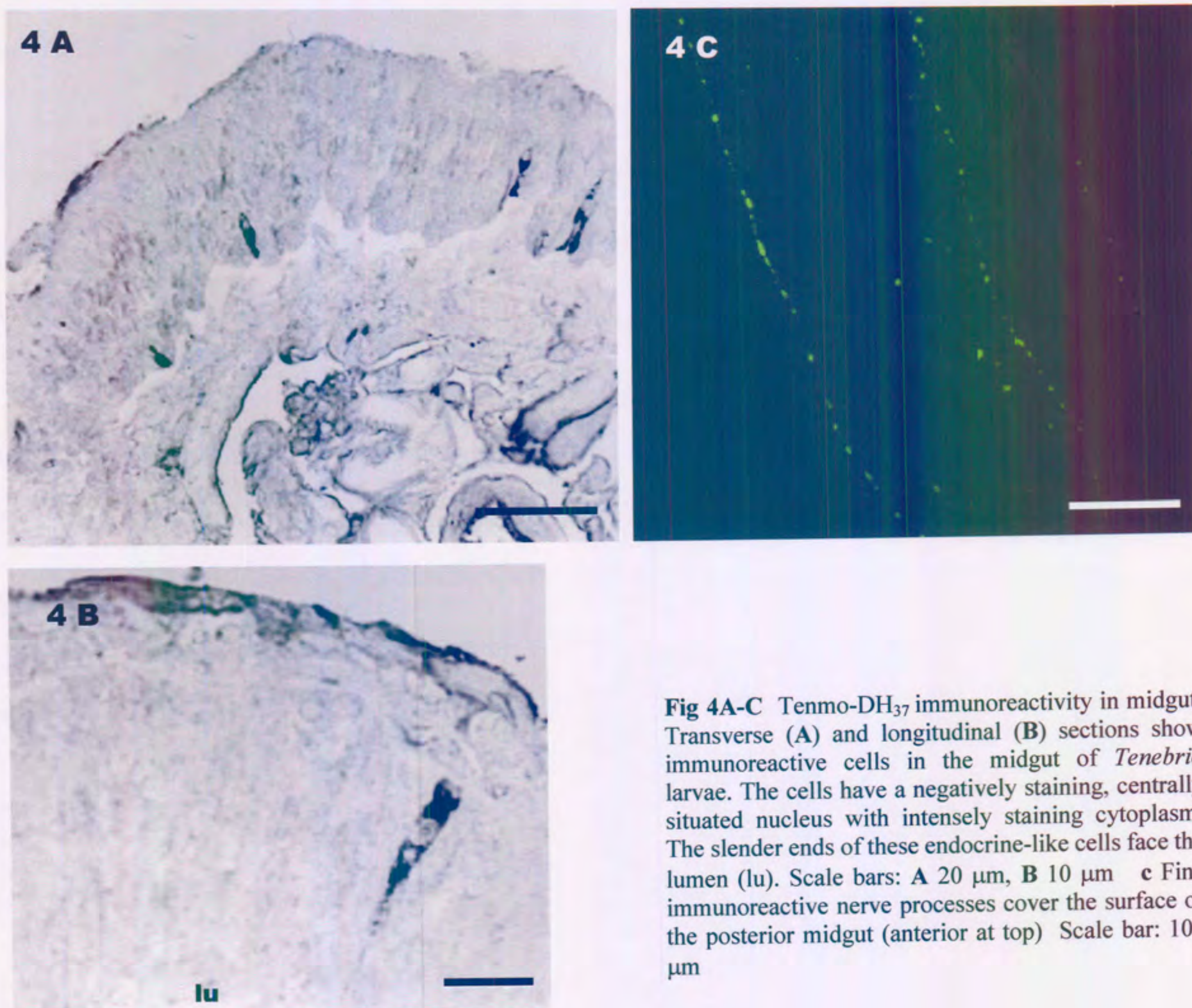

Fig 4A-C Tenmo-DH_{37} immunoreactivity in midgut. Transverse (**A**) and longitudinal (**B**) sections show immunoreactive cells in the midgut of *Tenebrio* larvae. The cells have a negatively staining, centrally situated nucleus with intensely staining cytoplasm. The slender ends of these endocrine-like cells face the lumen (lu). Scale bars: **A** 20 μm, **B** 10 μm **c** Fine immunoreactive nerve processes cover the surface of the posterior midgut (anterior at top) Scale bar: 100 μm

Discussion

This immunocytochemical study shows Tenmo-DH_{37}-immunoreactive neurosecretory cells in the pars intercerebralis of the brain, with axons projecting to the corpora cardiaca, where they end in varicose processes. The corpus cardiacum is an important neurohaemal organ and I have shown intense staining over the entire corpora cardiaca. This staining pattern in brain and corpora cardiaca is similar to that seen in previous studies where immunocytochemistry has been used to localise CRF-related diuretic peptides in *Locusta migratoria* (Patel et al. 1994) and *Manduca sexta* (Veenstra and Hagedorn 1991). In addition, antisera to Locmi-DH have demonstrated similar distribution of CRF-related peptides in adult *Musca domestica* (Iaboni et al. 1998) and in *Rhodnius prolixus* (Te Brugge et al. 1999). However, Tenmo-DH_{37} antiserum yielded negative results when tested on brain and corpora cardiaca of *Locusta migratoria*, reflecting the specificity of the antiserum. This may be because Locmi-DH terminates in a hydrophobic isoleucinamide compared to the more hydrophilic leucylasparagine-OH C terminus of Tenmo-DH_{37} (Table 1). The importance of the C terminus for activity is also demonstrated by the fact that, unlike the CRF-related diuretic hormones of other insect species (Audsley et al. 1995), synthetic Tenmo-DH_{37} does not stimulate tubules of *Manduca sexta* (Furuya et al. 1995). The specificity of the Tenmo-DH_{37} antiserum is further evident from the administration of Tenmo-DH_{37} together with the antiserum in the fluid secretion assay. In the fluid secretion experiment a 0.2 nM concentration of Tenmo-DH_{37} increased the fluid secretion rate by approximately 180%. This effect was immediate and maximum stimulation was obtained after 60 min. Combined administration reversed the stimulatory effect of the diuretic peptide on isolated tubules, effectively decreasing secretion rates to control levels after 60 min. It appears that the Tenmo-DH_{37} peptide has a stronger affinity for the antiserum than for the Tenmo-DH_{37} receptor on the Malpighian tubule cells.

The intense staining seen in the posterio-lateral neurosecretory cells in the abdominal ganglia of *Tenebrio* corresponds to the distribution of CRF-related diuretic hormones in *L. migratoria* (Patel et al. 1994; Thompson et al. 1995) and *M. sexta* (Chen et al. 1994). These bilaterally arranged cell bodies project processes out of the ventral nerve to their

respective neurohaemal organs. Axons exiting the ventral nerves stain in a varicose fashion that is characteristic of a neurohaemal release site (Fig 3A, B). A similar staining pattern was found in *Musca domestica* for a leukokinin-like peptide (Iaboni et al. 1998). The abdominal nerves of *R. prolixus* have long been considered to be sites of 'diuretic hormone' release (Maddrell 1966). The intense immunoreactive staining found in the ventral nerves of *Tenebrio* larvae is consistent with these findings. Moreover, the dense plexus of immunoreactive material in the terminal abdominal ganglion of *Tenebrio* (Fig. 3D, E) resembles the staining in the corpora cardiaca, suggesting the terminal abdominal ganglion as a possible neurohaemal release site.

The immunoreactive cells found in the epithelium of the midgut show the morphology of endocrine cells. The cytoplasm of these triangular shaped cells is packed with Tenmo-DH_{37}-immunoreactive material. Positive staining for CRF-related peptides has previously been detected in midguts of *Aedes aegypti* (Veenstra et al. 1995) and *Schistocerca gregaria* (Montuenga et al. 1989), and in the ampullae where Malpighian tubules of *Locusta migratoria* connect to the midgut-hindgut junction (Montuenga et al. 1996). The physiological role of these diuretic peptides in the midgut is not known. Where tenebrionid beetles are concerned, *Onymacris plana* and *Tenebrio molitor* inhabit dry environments and have no need for diuresis: however, brain and corpora cardiaca extracts from these insects have potent stimulatory effects on fluid secretion by their Malpighian tubules (Nicolson and Hanrahan 1986; Nicolson 1992). *In vivo* experiments involving injections of dye and corpora cardiaca extracts indicate that, when fluid is secreted rapidly by Malpighian tubules of these tenebrionids, it is directed to the midgut for recycling to the haemolymph (Nicolson 1991; U.I.M. Wiehart, unpublished data). The immunoreactive cells found in the midgut of *Tenebrio* may therefore be responsible for this recycling by rendering the gut epithelium more permeable. The effect of such recycling is that in these insects diuretic hormones, which should rather be termed clearance hormones, increase fluid secretion by the Malpighian tubules without affecting water balance of the insect.

Recycling of water also occurs *via* the rectal complex in Coleoptera and larval Lepidoptera. Audsley et al. (1993) found that Manse-DH increases salt transport in everted rectal sacs of *Manduca sexta*, with water following in a lumen to haemolymph direction, so that the effect of the diuretic peptide is antidiuretic, increasing fluid reabsorption. It is possible that diuretic peptides of *Tenebrio* may also stimulate those portions of the Malpighian tubules that lie within the rectal complex: considerable immunoreactivity to Tenmo-DH_{37} was evident in the terminal abdominal ganglion, although I did not examine the rectal complex itself for such immunoreactivity. In a detailed study of leucokinin and diuretic hormone distribution in both larval and adult *Manduca sexta*, Chen et al. (1994) found that neurons immunoreactive to both peptides projected to neurohaemal release sites within the rectal complex.

In this study I show the extensive distribution of a single diuretic peptide, Tenmo-DH_{37}, in the CNS and midgut of *Tenebrio*. A mechanism whereby Malpighian tubule secretion is controlled by at least two independent diuretic hormones, one a CRF-related peptide and the other an insect kinin, acting synergistically, was suggested by Coast (1995) and has been investigated by numerous authors (e.g., O'Donnell et al. 1996, 1998). Co-localization of leucokinins and the species-specific CRF-related diuretic peptides has been demonstrated in abdominal ganglia of *Manduca sexta* and *Locusta migratoria* (Chen et al. 1994; Thompson et al. 1995). The latter authors suggested that the co-localization of these peptides may be conserved among insect species. However, Iaboni et al. (1998) found separate populations of imunoreactive cells when testing antisera to Locmi-DH and leucokinin I in *Musca domestica*. Recently, Eigenheer et al. (2002) have isolated a potent antidiuretic peptide from *Tenebrio* pupae. This peptide inhibits fluid secretion by isolated tubules at femtomolar concentrations, and is the first endogenous insect antidiuretic peptide acting directly on Malpighian tubules to be sequenced. The possibility exists that the coordinated release of Tenmo-DH_{37} and the antidiuretic peptide creates a sensitive and potent mechanism for the control of fluid secretion. It would be interesting to investigate the possible co-localization of these peptides to substantiate this hypothesis.

Acknowledgments. I thank David Schooley (University of Nevada, Reno, USA) for the gift of Tenmo-DH_{37}.

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Paper 3

Isolation, Identification and Localization of a Second Beetle Antidiuretic Peptide

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Peptides (2003) (in press)

Contribution of co-authors other than supervisor(s)

R.A. Eigenheer conjugated the antidiuretic peptide to a protein.

K.M. Schegg sequenced and synthesized the antidiuretic peptide.

J.J. Hull assisted by doing the cyclic AMP determinations.

All the immunocytochemical work was done in Prof. Schoofs laboratory.

Isolation and synthesis of the antidiuretic peptide Tenmo-ADFb was done in Prof. Schooleys laboratory.

Note: **Paper 3** is written in the first person for thesis purposes

Abstract

A peptide that inhibits fluid secretion by the Malpighian of *Tenebrio molitor* tubules was isolated from head extracts of this insect. This second antidiuretic factor, ADFb, like the previously published ADFa, works through cyclic GMP as a second messenger. It has primary structure Tyr-Asp-Asp-Gly-Ser-Tyr-Lys-Pro-His-Ile-Tyr-Gly-Phe-OH with an EC_{50} of approximately 240 pM in a fluid secretion assay. This peptide is now the second sequenced endogenous insect ADF which inhibits Malpighian tubule fluid secretion. Immunohistochemical techniques show that the peptide is localized in the brain; it appears to be produced mainly in two pairs of bilaterally symmetrical cells in the protocerebrum.

Keywords: Water balance, cyclic GMP, fluid secretion, antidiuretic factor, *Tenebrio molitor*, Malpighian tubules.

Introduction

Insect osmoregulation requires diuretic and antidiuretic neurosecretory hormones which control ion transport in the excretory system (Coast, 1998). Several families of diuretic factors have been isolated which promote fluid secretion from the Malpighian tubules of the insect (Coast, 1996; Furuya et al., 2000; Wheeler and Coast, 1990). Most insect antidiuretic factors characterized to date stimulate fluid reabsorption in the hindgut (Audsley et al., 1992; Fournier and Girardie, 1988; Liao et al., 2000; Phillips et al., 1996; Proux et al., 1984); studies of antidiuretic factors inhibiting fluid secretion from the Malpighian tubules are more rare (De Decker et al., 1994; Laenen et al., 2001; Lavigne et al., 2001; Petzel and Conlon, 1991; Spring et al., 1988). Before the isolation in this laboratory of *T. molitor* ADFa (Tenmo-ADFa) (Eigenheer et al., 2002), cardioacceleratory peptide 2b (CAP_{2b}) was the only insect peptide characterized that elicits cyclic GMP production to affect Malpighian tubule fluid secretion. CAP_{2b}, which was originally isolated as a myotropin from *Manduca sexta* (Heusmann et al., 1995) was reported to elevate NO and cyclic GMP to stimulate diuresis in *Drosophila melanogaster* (Davies et al., 1995). In contrast, CAP_{2b} was characterized as antidiuretic in the bloodsucking insect *Rhodnius prolixus*; CAP_{2b} seems to function through cyclic GMP as a second messenger, independent of NO in this species (Quinlan et al., 1997). Recently, the highly potent peptide Tenmo-ADFa,

which also works through cyclic GMP without the involvement of NO to cause a decrease in fluid secretion was isolated and identified (Eigenheer et al., 2002); ADFa is 108 times more potent than CAP_{2b} at inhibiting fluid secretion in *T. molitor*.

This study reports the purification from heads of *T. molitor* of the second insect ADF (ADFb) which causes antidiuresis in the tubules of this species, as well as the peptide's sequence analysis, synthesis, and immunohistochemical localization. A synthetic replicate of this peptide is extremely potent, with an EC_{50} value in a fluid secretion assay of 240 pM. This ADF appears to utilize cyclic GMP as a second messenger, like ADFa and CAP_{2b}. Immunocytochemical evidence indicates that this peptide is localized in two pairs of bilaterally symmetrical neurosecretory cells in the protocerebrum of *T. molitor*, and also shows very strong staining in the nerve cord. These data strongly suggest that ADFb is a bona fide neuropeptide, rather than an artifact of proteolysis of *T. molitor* putative cuticle protein TmPCP 9.2 (Baernholdt and Andersen, 1998). The C-terminus of TmPCP 9.2 has 100% sequence identity to this neuropeptide.

Methods

Experimental animals

T. molitor used for peptide isolation and second messenger assays were reared as described earlier (Eigenheer et al., 2002). Heads for extraction of peptides were removed and frozen immediately in liquid N_2, then stored at 80 °C. Malpighian tubules for second messenger bioassays were removed from adults 0 - 2 hrs after emergence. Last instar larvae used for fluid secretion assays and immunohistochemistry (IHC) were kept under crowded conditions at room temperature (20-23 °C) and fed on a diet of bran and apple.

Second messenger bioassays

Cyclic AMP-elevating activity from synthetic ADFb was detected with a competitive enzyme-linked immunoassay (EIA), as described previously (Eigenheer et al., 2002). Aliquots of chromatographic fractions were prepared as for ADFa (Eigenheer et al., 2002). Cyclic GMP-elevating activity in fractions from purification steps was

detected with a different competitive EIA, as described earlier (Eigenheer et al., 2002).

Biological (fluid secretion) assay.

A modified Ramsay assay was employed for measurement of fluid secretion by *T. molitor* Malpighian tubules as previously described (Nicolson, 1992). Secretion of tubules was measured in Ringer's solution (control), then subsequently with either Ringer's solution or ADF plus Ringer's. Antidiuretic activity was calculated as % inhibition of maximum basal fluid secretion. Five to eight replicates were done for each experiment.

Extraction and isolation from heads.

The peptide ADFb was isolated as a second active fraction during the isolation of ADFa from a 1500 head equivalent aliquot of 10,000 extracted pupal *T. molitor* heads (Eigenheer et al., 2002). During the purification, all reversed-phase liquid chromatography (RPLC) was done with a Hewlett-Packard model 1050 pumping system and UV detector, with Rheodyne 7125 loop injector. 500 head equivalents from the cation exchange step were loaded onto a 2.1 x 150 mm C_8 Zorbax reversed-phase column (Eigenheer et al., 2002), then equilibrated with 0.1 % aqueous TFA before and after loading of samples. The bound fractions were eluted with a 60 min 0-57% CH_3CN in 0.1% TFA linear gradient at 0.2 ml/min, at ambient temperature. The active ADFb fraction eluted at 22.5% CH_3CN, as compared with 17% CH_3CN for ADFa (Eigenheer et al., 2002). This gradient was repeated twice, each time with 500 head equivalents from the previous step; active fractions were combined and diluted fivefold with 0.1% aqueous TFA before the next step. These pooled fractions were then loaded onto a 1 x 150 mm C_4 Vydac column at 60 l/min, at ambient temperature, then equilibrated as before with 0.1% TFA. Bound fractions were eluted with the same linear gradient, with the active ADFb fraction eluting at 16% CH_3CN, *vs.* 13.5% for ADFa (Eigenheer et al., 2002). This fraction was again diluted fivefold with 0.1% aqueous TFA, then loaded onto a 1 x 150 mm Zorbax SB cyano column at 60 l/min (Eigenheer et al., 2002) and equilibrated with 0.1% aqueous TFA. The bound fractions were eluted as in the preceding RPLC steps (same gradient and temperature), with ADFb eluting at 26% CH_3CN, *vs.* 19% for ADFa (Eigenheer et al., 2002). All RPLC fractions were separated by peak shape, and collected in 1.5 ml

"lubricated" (Corning Costar) tubes, which were prewashed with CH_3OH to prevent sticking. 1500 *T. molitor* head equivalents yielded approximately 200 pmol of pure ADFb peptide.

Peptide microanalysis and synthesis

Sequence analysis of the purified ADFb was performed using automated Edman degradation on an Applied Biosystems Procise 492 sequencer. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry was performed on a Bruker Proflex Plus instrument. ADFb peptides were synthesized in both amidated and free acid carboxyl terminus forms, using an Applied Biosystems 431A synthesizer and *N*-9-fluorenylmethoxycarbonyl (FMOC) chemistry. Synthesis was done in 1-methyl-2-pyrrolidinone in the presence of dicyclohexylcarbodiimide and 1-hydroxybenzotriazole for FMOC-amino acid activation. Amide and acid C-terminal forms of the peptide were synthesized simultaneously using 50 μmol each of p-hydroxymethylphenoxy (Wang, Applied Biosystems) and Rink MBHA amide (Novabiochem) resins. These were then separated by RPLC; the separations were done on a 20 x 250 mm (300) C_8 YMC column. The initial gradient used for separation of ADFb amide from acid was 0-57% ethanol (EtOH) in 60 min with 0.1% TFA maintained throughout. A higher resolution separation was done with 0-14.25% EtOH in 10 min, then 14.25-57% EtOH in another 90 min.

Conjugation and antiserum production

ADFb was synthesized with a Cys C-terminal extension, [Cys^{14}]ADFb. This allowed conjugation to the ε-amino group of Lys residues in ovalbumin using the sulfo-GMBS bifunctional crosslinking reagent (Pierce). The Cys was added to the C-terminus, because the N-terminal part of the peptide appeared to be most antigenic, according to analysis with MacVector 4.0. Ovalbumin has 20 Lys residues, but not all may be accessible to solvent, so a ratio of slightly greater than 20/1 for peptide/ovalbumin was used for conjugation to assure multiple copies of peptide for each conjugate. Sulfo-GMBS contains a thiol-reactive N-linked maleimide group on one end and an amine-reactive N-hydroxysuccinimide (NHS) ester at the other end. 10 mg ovalbumin (Sigma) was dissolved in 2 ml 50 mM HEPES buffer, pH 8.0; to this was added a 30-fold molar excess of sulfo-GMBS with stirring. The mixture was allowed to react overnight (8 hrs) in the cold room (4 °C), then quenched with 100 ml 1 M Tris, pH

8.0; the quench proceeded for 1 hr to assure completeness. This solution was dialyzed against 1 liter 0.15 M phosphate-buffered saline (PBS), pH 7.5, three times (at least 4 hr total). To the ovalbumin solution was added 7.5 mg of [Cys^{14}]ADFb; the mixture was allowed to react 4 hrs in the cold room, then quenched with 100 1 1 M cysteine for 1 hr. After final quench, this solution was dialyzed against 1 liter of 1% acetic acid three times. Antibodies to the ADFb conjugate were raised in rabbits, and test bleeds evaluated by ELISA. After good titers to the conjugate of ADFb were obtained, production bleeds were performed.

Immunocytochemical methods

Tissue sections of the CNS (brain, suboesophageal ganglion, ventral nerve cord), corpora cardiaca, midgut and rectal complex of *T. molitor* larvae were prepared as previously described (Wiehart et al., 2002). Sections were stained using the indirect immunoperoxidase method (IIPO). Briefly, after preincubation with normal goat serum, the tissue sections were incubated overnight with the primary antibody at a dilution of 1:500. Following a rinse with 50 mM Tris-buffered saline (TBS), slides were incubated in a 1:200 dilution of goat anti-rabbit peroxidase conjugated secondary antibody. The peroxidase-conjugated antibodies were used for visualization with the staining reaction described below.

Whole-mount immunofluorescence

The method used for whole-mount immunofluorescence has been described previously (Wiehart et al., 2002). Nervous tissue, midguts and rectal complexes were permeabilized in 4% Triton X-100, containing 2% normal porcine serum and 2% bovine serum albumin. Tissues were incubated with the primary Tenmo-ADFb antiserum at a dilution of 1:500 whereafter they were washed and placed in FITC (fluorescein isothiocyanate)-conjugated swine anti-rabbit immunoglobulins (DAKO) secondary antibody. All preparations were mounted in 90% glycerol containing 0.1% paraphenylenediamine and viewed by confocal microscopy on a Zeiss LSM 410 instrument.

Serum specificity

Specificity of the antiserum was determined by application of the pre-immune rabbit

antiserum taken from the animal that produced the primary antiserum (Tips et al., 1997). To avoid false positive results the primary antiserum was pre-absorbed with the Tenmo-ADFb peptide (500 g/ml) overnight at 4 °C and applied to whole-mount and serial section samples. A dot immunobinding assay (Salzet et al., 1997) was used to characterize the antisera. This assay and the immunohistochemistry methods have been described in detail elsewhere (Wiehart et al., 2002). Briefly, aliquots of Tenmo-ADFb were spotted onto a nitrocellulose membrane in a dilution series. Baked membranes were blocked with skimmed milk in 50 mM Tris-buffered saline (TBS) and incubated overnight at 4 °C with the Tenmo-ADFb antiserum (diluted 1:1000 in TBS). After washing, the membranes were incubated with peroxidase-conjugated goat anti-rabbit IgG (1:200) after which the immunoreactive spots were visualized with 0.01% hydrogen peroxide as an enzyme substrate and 12.5 g/ml 3-3 - diaminobenzidine as a chromogenic reagent.

Results

Isolation and Identification

Edman sequence analysis of the purified ADFb indicated a primary structure of YDDGSYKPHIYGF, with a theoretical monoisotopic mass of 1560.688. Matrix-assisted laser desorption ionization time of flight (MALDI-TOF) mass spectrometry of the biological peptide indicated a mass of 1560.33 which is consistent with the theoretical mass for ADFb. Peptides having this sequence were synthesized in amidated and free acid carboxyl terminus forms, then separated by RPLC. The second RPLC separation described in Methods, which was done with a gradient of 0-14.25% EtOH in 10 min, then 14.25-57% EtOH over 90 min, gave a better (closer to baseline) separation of ADFb amide and acid forms. Native (biological) ADFb was coinjected separately with synthetic amide and acid ADFb on a 1 mm C_4 Vydac reversed-phase column; the native peptide coelutes with the free acid form of synthetic ADFb (Fig. 1), confirming the sequence analysis and demonstrating a complete structure of YDDGSYKPHIYGF-OH. The free acid structure was also confirmed by analysis of mixed native and synthetic peptide by MALDI-TOF mass spectral analysis. This sequence has been deposited in the SWISS-PROT database as accession #P83109. Tenmo-ADFb does not significantly resemble any known biologically active peptide,

unlike ADFa, which bears some resemblance to the portion of human and rabbit big endothelin I cleaved off into an inactive piece on proteolysis of endothelin I (Eigenheer et al., 2002). ADFb is identical to the 13 C-terminal residues of *T. molitor* putative cuticle protein 9.2 (Fig. 2). This cuticle protein (TmPCP 9.2) was purified using standard protein isolation techniques, and no genomic sequence data is available for it (Baerenholdt and Andersen, 1998).

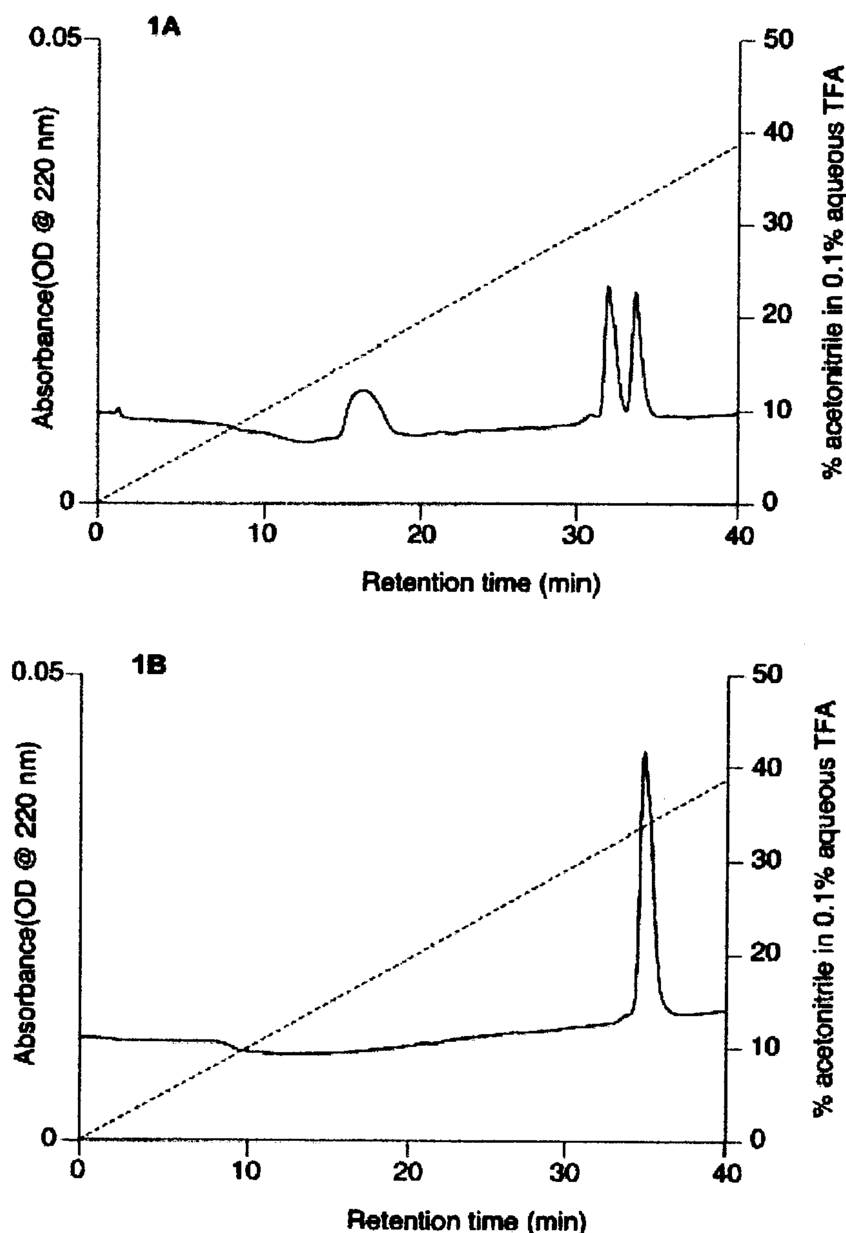

Fig. 1.(A) ADFb biological (natural) peptide coinjected with synthetic ADFb amide and separated by RPLC; 1 mm Zorbax cyano column, eluted at 50 µl/min with a gradient of 0 to 57% acetonitrile over 60 min with 0.1 % TFA maintained throughout. Detector was set to 220 nm and 0.02 AUFS. **(B)** ADFb biological (natural) peptide coinjected with synthetic ADFb acid on 1 mm Zorbax cyano column; eluted with same gradient and conditions. The peptide is retained slightly longer than in Fig. 1(A) due to instrumental variations.

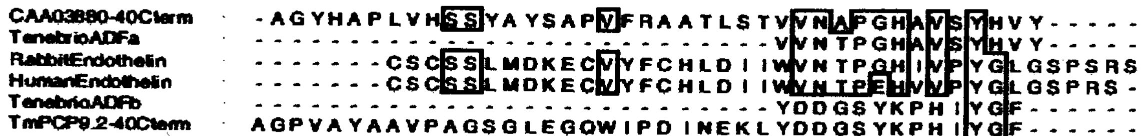

Fig. 2. Sequence alignment of *T. molitor* (Tenmo) ADFa and b, *T. molitor* cuticle proteins CAA03880 (40 C-terminal residues only) and TmPCP 9.2 (40 C-terminal residues only), and rabbit (r) and human (h) big endothelin I, aligned using Clustal W V. 1.8 on sequences from the SWISS PROT database. Identical residues are boxed. Endothelin I is cleaved by endothelin converting enzyme at the W-V bond; the homology to Tenmo-ADFa occurs in the inactive part of big endothelin I, on the C-terminal side of the cleavage site.

Biological Activity

A cyclic GMP second messenger assay was used to monitor activity of our antidiuretic factor during purification and a modified Ramsay fluid secretion assay was used to demonstrate the biological activity and its dose dependence. ADFb caused a reversible and dose-dependent decrease in fluid secretion by *T. molitor* tubules (Fig. 3) with an EC_{50} 240 pM, compared with an EC_{50} of 10 fM for ADFa (Eigenheer et al., 2002). Like ADFa, ADFb showed significant desensitization of response at higher concentrations (not shown), possibly due to receptor down-regulation or internalization. As with ADFa, an initial experiment was done to determine if ADFb has effects on cyclic AMP titer in *T. molitor* Malpighian tubules which were stimulated by the potent CRF-like diuretic hormone DH_{37} from *T. molitor* (Furuya et al., 1995). There was a significant difference between tubules treated with 1 pM ADFb and 10 nM DH_{37} *vs.* the DH_{37} alone, as measured by a student t-test with a p-value of 0.015.

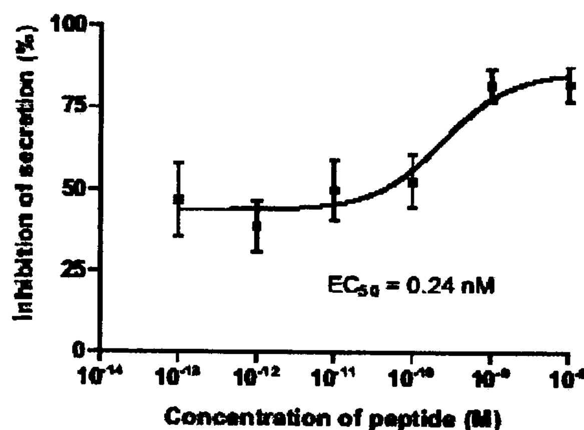

Fig. 3. Dose-response curve for Tenmo-ADFb with Malpighian tubules of *T. molitor*. Fluid secretion was measured at various concentrations of hormone and the percent inhibition determined. The data are given as % inhibition of secretion – standard error, and were fitted by non-linear regression analysis using Prism¤ 3.0. Basal secretion is determined on Malpighian tubules initially in control solution (Ringer's); subsequently this is replaced with more Ringer's or Ringer's containing antidiuretic factor. Some loss of basal secretion is always observed after fluid replacement; this accounts for the response never reaching zero inhibition.

Partial purification of ADFb from nervous tissue

Due to the structural identity between ADFb and the *T. molitor* cuticle protein, I wished to demonstrate that ADFb was a unique peptide which could be found in tissues lacking in cuticle proteins. To achieve this, peptides from neuroendocrine tissues, the nerve cord and brain, were extracted and partially isolated using a scaled down version of that protocol described in Methods for purification of ADFb. The partially purified peptides from 5 *T. molitor* nerve cords were chromatographed on a 1 mm C_4 Vydac column, then fractions were collected and assayed for cyclic GMP-elevating activity. A zone with retention time similar to that of synthetic ADFb acid, which was chromatographed subsequently on the same column, increased production of cyclic GMP in the Malpighian tubules. In addition, peptides from 10 *T. molitor* brains were extracted and partially purified, and these were separated on the same column, with synthetic peptides running subsequently. Again, the result was a cyclic GMP-stimulating active zone with retention behavior similar to that of synthetic ADFb acid.

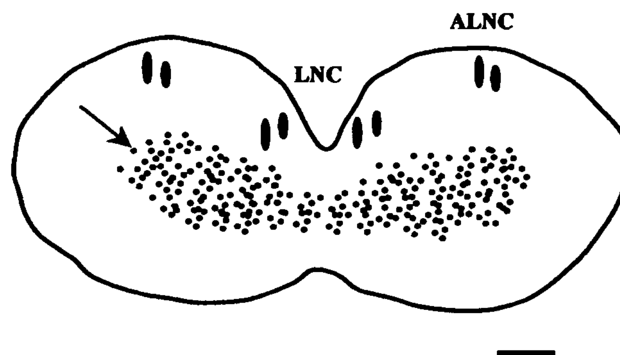

Fig. 4. Schematic illustration of Tenmo-ADFb immunoreactivity in the brain of *Tenebrio molitor*. Lateral neurosecretory cells (LNC) and anterior-lateral neurosecretory cells (ALNC) in the pars intercerebralis of the protocerebrum project processes posteriorly to the proposed neurohaemal release site (arrow). Scale: 100 μm.

Immunohistochemistry

To further demonstrate the presence of ADFb in non-cuticular tissues, the peptide was synthesized with a Cys C-terminal acid residue extension, [Cys^{14}]ADFb, conjugated it to ovalbumin with the bifunctional crosslinking reagent sulfo-GMBS (Pierce), then antibodies were raised in rabbits. Immunohistochemistry was performed with pre-treated, sectioned tissues of *T. molitor*. Strong staining in neuroendocrine cells was expected due to the abundance of both antidiuretic peptides in the insects; only 1500

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heads were necessary to purify both factors. Fig. 4 illustrates the bilaterally symmetrical distribution of Tenmo-ADFb immunoreactive cells in the cerebral lobes. Two pairs of lateral neurosecretory cells (LNC) located anteriorly in the protocerebrum stained strongly along with another pair of neuroreactive cells located anterior and lateral (ALNC) to the LNC. These cell bodies (diameter 20-25 μm) project axons posteriorly to enter a plexus of blebby staining neuroreactive material, characteristic of a neurohaemal release site (Fig. 5A and B).

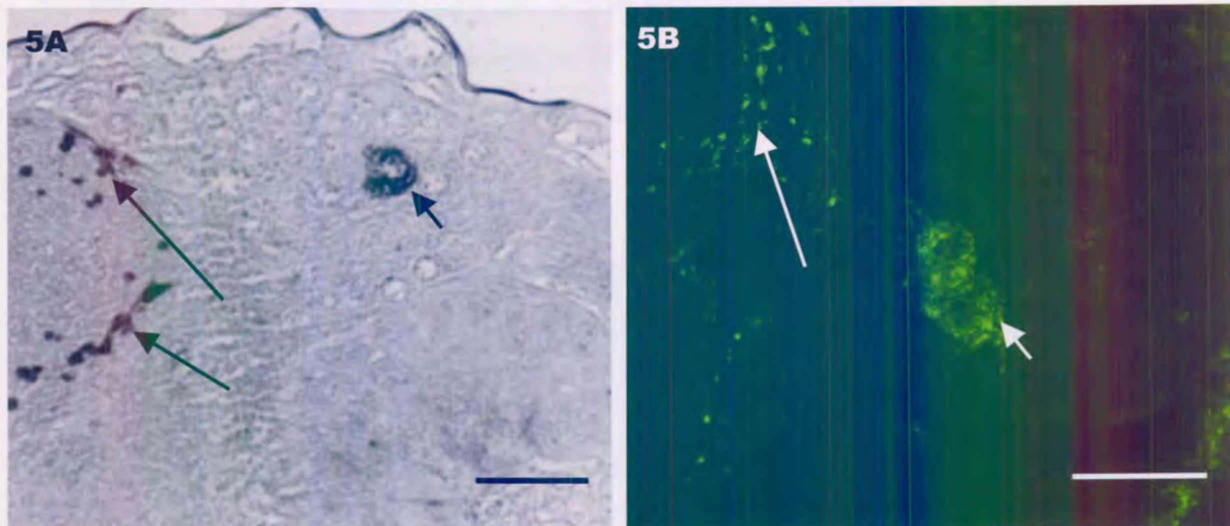

Fig. 5. Tenmo-ADFb neuroreactive cells in the brain. Strongly positive staining cells in the protocerebrum (short arrow) and intense staining immunoreactive material (long arrows) indicating a possible neurohaemal release site as determined by (**A**) IIPO method (scale: 50 μm) and (**B**) FITC immunofluorescence (scale: 25 μm).

The staining of this plexus was extensive and neuroreactive material was found throughout the midline of the brain, perhaps indicating appreciable storage of the peptide. No staining was found in the deuto- and tritocerebrum or in the corpora cardiaca, which is a major neurohaemal organ for the release of brain neurosecretory products into the hemolymph (Wiehart et al., 2002). ADFb immunoreactive staining was also present in the ventral nerve cord of the insect. The ventral nerve cord of *T. molitor* larvae consists of a suboesophageal ganglion (SOG), three thoracic ganglia and eight abdominal ganglia. The paired connectives are separated between the SOG and the three thoracic ganglia, but fused between the abdominal ganglia. Immunoreactive material was visible in all the ganglia except the SOG. The immunoreactive staining and blebbed axons followed no clear pattern, but were

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concentrated in the midline of each ganglion (Fig. 6A and B). No neurosecretory cells could be seen in any of the ganglia. However, immunoreactive cells were found in the connectives (Fig. 6C) and in the median nerve situated between the connectives of the metathoracic ganglion and the first abdominal ganglion. The cells in the median nerve were 15-20 μm with brightly staining granules (Fig. 6D) compared to those found in the connectives, which were smaller at approximately 10 μm. Nonspecific staining was found along the hemolymph side of both midgut and rectal complex: this staining was not abolished after preabsorption of the antiserum with Tenmo-ADFb. The cuticle which lines the rectal complex stained only on the surface, owing to its impermeable structure.

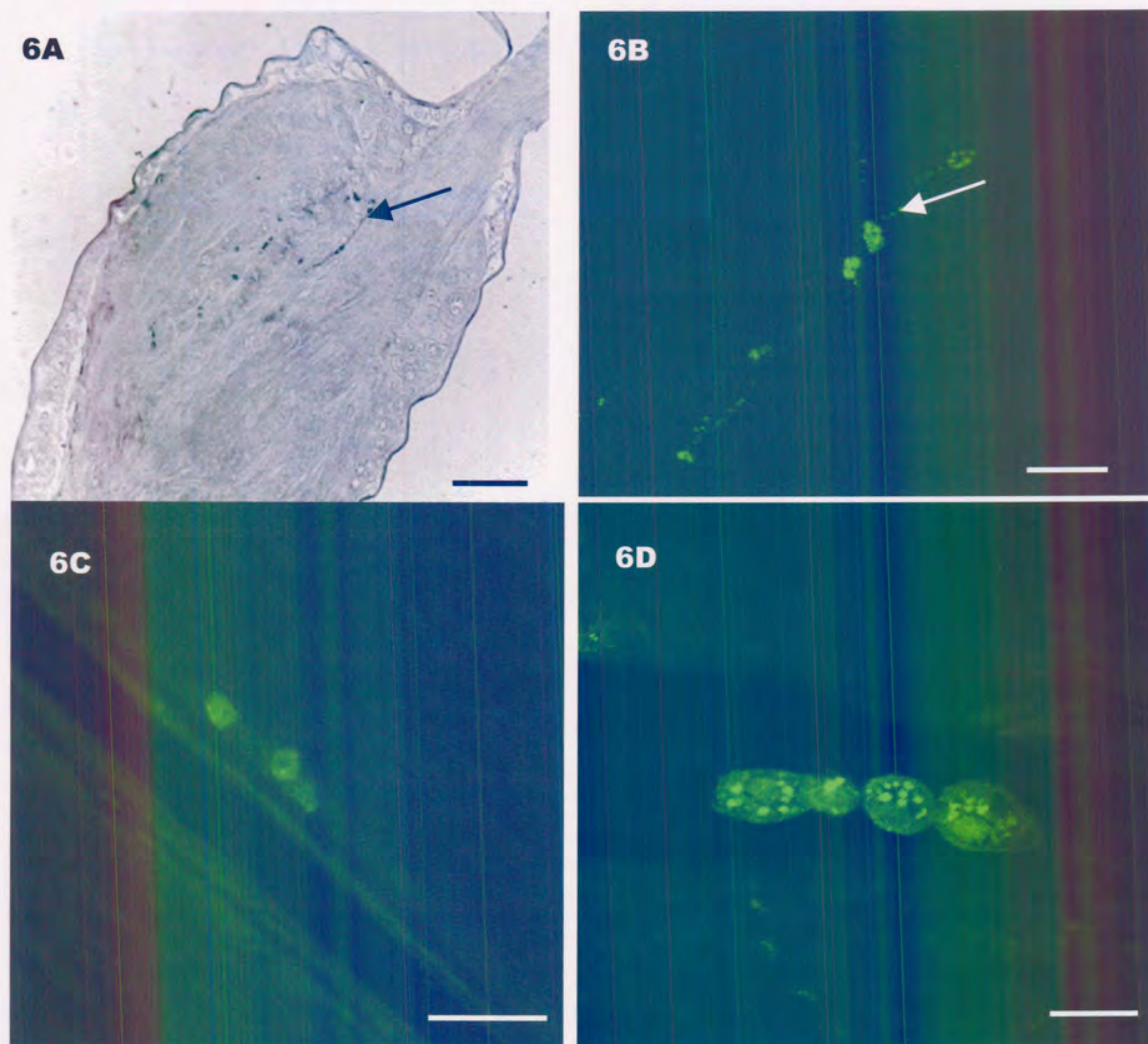

Fig. 6 **(A-D)** Tenmo-ADFb immunoreactivity in the ventral nerve cord. **(A,D)** Fine, centrally located immunoreactive nerve processes are present in all the ganglia with exception of the suboesophageal ganglion. **(C)** Tenmo-ADFb immunoreactive cells in the connectives and **(D)** in the median nerve between the metathoracic ganglion and the first abdominal ganglion. Scale: **(A,C)**, 50 μm (IIPO method) and **(B,D)**, 10 μm (FITC immunofluorescence).

Discussion

This paper documents the second antidiuretic factor sequenced from the Coleoptera, both from *T. molitor*. This peptide, Tenmo-ADFb, again exerts its effects through the second messenger cyclic GMP to cause a decrease in fluid secretion of *T. molitor* Malpighian tubules. Tenmo-ADFb is a very potent peptide, with an EC_{50} 240 pM, and could represent a peptide important for water balance in this species, despite the fact that it is approximately 24,000 times less potent than ADFa. The fact that ADFb stains immunologically in both the nerve cord and brain of *T. molitor*, and the chromatographic characteristics and activity of crude extracts from these tissues, lend credence to this possibility.

ADFb, like ADFa, lowers the cyclic AMP titer in *T. molitor* Malpighian tubules which have been stimulated with *T. molitor* DH_{37}. This antagonistic effect between cyclic AMP and cyclic GMP is not unprecedented, with the tubules of *R. prolixus* being the most relevant example (Quinlan and O'Donnell, 1998). Wiehart et al. (2002) have recently extended these studies using *T. molitor* tubules, and have shown an antagonism not only between the nucleotides cyclic AMP and cyclic GMP but also between the endogenous CRF-like DH *T. molitor* DH_{37} and Tenmo-ADFa.
However, given the very large difference in EC_{50} values of ADFa and ADFb (24,000 X) and the lack of mutual sequence similarity, it is possible that antidiuretic activity may not be the primary function of ADFb. Even the relatively "low" activity (EC_{50} = 0.24 nM) of ADFb makes this peptide still more potent than the majority of CRF-like DH (Audsley et al., 1995), but neuropeptides are generally pleiotropic. Perhaps future comparative studies in the immunohistochemical localization of the two peptides may allow some guesses about alternative targets for these peptides. Despite the apparent high abundance of these antidiuretic peptides, as gauged by the amount isolated from 1500 head equivalents, the immunocytochemical evidence does not point to any classical storage sites for them in neuroendocrine tissues. The corpora cardiaca, which usually stain strongly for CRF-like DH (Audsley et al., 1997; Iaboni et al., 1998; Te Brugge et al., 1999; (Wiehart et al., 2002; Zitan et al., 1995), showed no cross-reactivity to our ADFb antisera. Perhaps the "blebby material" in the brain represents storage sites.

As described previously (Eigenheer et al., 2002), it was demonstrated that the nitric oxide (NO) donors sodium nitroprusside and *S*-nitroso-L-Cys have no effect on the amount of cyclic GMP secreted by *T. molitor* Malpighian tubules. It was also demonstrated that neither N^G-monomethyl-L-Arg (L-NMMA) nor *S*-methylisothiourea would block the cyclic GMP stimulation from crude peptide extract (Eigenheer et al., 2002). These experiments suggest that NO does not act as a second messenger in this system.

The fact that both ADFa and ADFb resemble structural proteins of the same species is unprecedented. It is unlikely that ADFa is merely cleaved from the cuticle protein; the cuticle protein precursor lacks a dibasic cleavage site where this peptide sequence begins; known neuropeptide precursors contain basic cleavage sites for KEX2 or furin-like enzymes (Veenstra, 2000). Unfortunately, no genomic precursor information is available for the protein TmPCP 9.2 that has a C-terminus identical to ADFb.

A recent paper describing the characterization of an antidiuretic factor from *Leptinotarsa decemlineata* (Lavigne et al., 2001) could point to some conservation of such factors in evolution. The factor described by these authors has hydrophobicity (reversed-phase retention characteristics) and apparent molecular weight similar to those of ADFa and ADFb.

The role of peptides which inhibit Malpighian tubule fluid secretion remains unclear. However, *T. molitor* are so efficient at removing water from food and retaining it, that their feces may actually have a lower water content than the dry bran they eat (Ramsay, 1964). If these antidiuretic factors are evolutionarily conserved among the Coleoptera, they could be used to develop a new family of pesticides to target beetle pests specifically.

Acknowledgements

This research was supported by the National Science Foundation (IBN-9602148), the National Institutes of Health (GM 48172), the Nevada Agricultural Experiment Station, the South African National Research Foundation, and a bilateral award

(Bil98/53) under the Flemish-South African agreement on scientific and technological cooperation. We thank P. Torfs for raising antibodies to the ADFb conjugate, and A. Van Lommel for the use of the confocal microscope.

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Paper 4

K^+ transport in Malpighian tubules of *Tenebrio molitor*: a study of electrochemical gradients and basal K^+ uptake mechanisms

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Journal of Experimental Biology (in press)

Note: **Paper 4** is written in the first person for thesis purposes.

Abstract

Malpighian tubules of *Tenebrio molitor* were isolated for intracellular measurement of basolateral (V_{bl}) and indirectly apical membrane potentials (V_{ap}). In control Ringer (50 mmol l^{-1} K^+, 140 mmol l^{-1} Na^+), V_{bl} was 24 mV, cell negative, and V_{ap} was 48 mV, cell negative with reference to the lumen. Ion substitution experiments involving K^+ and Na^+ indicated that both V_{bl} and V_{ap} were sensitive to the bathing K^+ concentration, with the change in V_{ap} being 60-77% that of V_{bl}. A ten-fold drop in bath [K^+] irreversibly decreased fluid secretion rates from 6.38 ± 0.95 (mean ± S.E.M.) to 1.48 ± 0.52 nl/min (n=8). In the presence of 6 mmol l^{-1} Ba^{2+}, a blocker of basal K^+ channels, fluid secretion rates reversibly decreased and the hyperpolarization of both V_{bl} and V_{ap} seen in 50 and 140 mmol l^{-1} K^+ indicated a favourable electrochemical gradient for basal K^+ entry. In 5 mmol l^{-1} K^+, Ba^{2+} induced two different responses: V_{bl} either hyperpolarized by approximately 10 mV or depolarised by approximately 14 mV, according to the electrochemical gradient for K^+, which was either inward or outward in low bath [K^+]. Rubidium, a 'permeant' potassium substitute, caused a hyperpolarization of V_{bl}, indicating the specificity of K^+ channels found in *Tenebrio* tubule cells. Other possible K^+ uptake mechanisms located in the basolateral membrane were investigated. Blocking of the putative electroneutral $Na^+/K^+/2Cl^-$ cotransporter by 10 µmol l^{-1} bumetanide reversibly decreased fluid secretion rates, with no detectable change in membrane potentials. Ouabain (1mmol l^{-1}), a Na^+/K^+-ATPase inhibitor, irreversibly decreased fluid secretion rates but had no effect on electrical potential differences either in the absence or presence of Ba^{2+}. The results implicate K^+ channels, the $Na^+/K^+/2Cl$ cotransporter and the Na^+/K^+-ATPase in basal K^+ and fluid transport of *Tenebrio* tubule cells.

Introduction

The mechanisms underlying ion transport in insect Malpighian tubules have been studied extensively in a number of species (for reviews see Maddrell and O'Donnell, 1992; Nicolson, 1993; Pannabecker, 1995; Beyenbach, 1995). Consensus has now been reached on a model for ion and fluid transport, which is driven primarily by an apical vacuolar-type H^+-ATPase. In Malpighian tubule cells the electrogenic transport of H^+ into the tubule lumen establishes a proton gradient and an electrical potential difference across the apical membrane, which drives movement of alkali cations from the cell to the lumen through apical Na^+/nH^+ and/or K^+/nH^+ antiporters. In addition the

proton pump generates a favourable electrical gradient for anion movement (Cl^-), which may be either transcellular or through the paracellular shunt (Beyenbach, 1995). With the exception of some insects (e.g., *Glossina*, Gee 1976; *Libellula*, Nicholls, 1985) that rather make use of sodium, potassium acts as the primary driving force of urine formation in Malpighian tubules. Blood-sucking species can switch from a mixed Na^+/K^+ in basal conditions to a preferentially Na^+ driven fluid secretion after a blood meal (e.g. *Rhodnius*, Maddrell, 1980).

Basolateral K^+ transport systems must permit adequate K^+ uptake to maintain fluid secretion. The existence of Ba^{2+} sensitive K^+ channels has been confirmed in tubules of *Onymacris* (Nicolson and Isaacson, 1987), *Formica* (Weltens et al., 1992), *Aedes* (Masia et al., 2000) and *Locusta* (Hyde et al., 2001). Uptake mechanisms for K^+ and/or Na^+ at the haemolymph side may differ according to the species. In tubules of *Formica polyctena*, alternative routes for basal K^+ entry appear to be implicated over different ranges of bathing saline [K^+]. In the presence of a high [K^+], entry occurs via high conductance, Ba^{2+} sensitive channels. At lower [K^+], K^+/Cl^- and/or $Na^+/K^+/2Cl$ co-transporters become functional (Leyssens et al., 1994; Van Kerkhove, 1994). Although there has been some controversy about the presence of a basolateral Na^+/K^+-ATPase in insect epithelia, this ouabain-sensitive electrogenic pump has been implicated in ion transport in *Locusta* (Anstee et al., 1986) and *Rhodnius* (Maddrell and Overton, 1988) tubule cells. However, fluid secretion rates of *Drosophila melanogaster* (Dow et al., 1994) and *Formica* (Leyssens et al., 1994) tubules remain unaffected by ouabain and thus, if present, the Na^+/K^+-ATPase does not appear to play a role in fluid secretion.

The present study focuses on possible K^+ uptake pathways across the basolateral membrane of *Tenebrio* tubule cells and demonstrates the impact of the apical membrane on passive K^+ uptake *via* the Ba^{2+}-sensitive K^+ channels. Fluid secretion rates and potentials across basolateral and apical membranes have been measured at different bath K^+ concentrations, and in the presence and absence of various blockers. The results obtained indicate the direction of the electrochemical gradient for K^+, the influence of the apical membrane potential on this gradient, and the relative importance of the alternative K^+ uptake routes. This study provides a basis for future studies in which (1) the nature of the K^+ channels involved in basolateral K^+ uptake is

further investigated and (2) the various K^+ uptake mechanisms are implicated as sites for regulation by endogenous factors.

Key words: K+ transport, K+ uptake, Malpighian tubules, *Tenebrio molitor*, K+ channel, Na+/K+/2Cl cotransporter, Na+/K+-ATPase, basolateral membrane, transepithelial membrane, apical membrane, fluid secretion rate, membrane potential.

Material and Methods

Animals

Tenebrio larvae used in this study were maintained in dry bran cultures at room temperature (20-23°C). The diet was supplemented weekly with apple as a source of moisture. Care was taken in selecting mealworms of similar size for all experiments.

Artificial salines

The composition of the bathing solution is summarized in Table 1. Solutions were freshly prepared each week, filtered through 0.22 µm Millipore filters and kept at 2 °C until used. The pH was measured daily before use. In experiments containing Ba^{2+}, NaH_2PO_4 was omitted from all salines to maintain constant osmolality and prevent precipitation. Control experiments in which NaH_2PO_4 was omitted showed no change in secretion rate or electrical profile. In low Na^+ experiments, salines contained a maximum of 6 mmol l^{-1} Na^+.

The following pharmacological substances were tested on Malpighian tubule preparations: barium chloride (Sigma), ouabain (Fluka), bumetanide (Sigma).

		A	B
	Control	(K^+ free)	(low Na^+)
NaCl	90	140	/
KCl	50	/	140
$MgCl_2$	5	5	5
$CaCl_2$	2	2	2
$NaHCO_3$	6	6	6
NaH_2PO_4	4	4	4
glycine	10	10	10
proline	10	10	10
serine	10	10	10
histidine	10	10	10
glutamine	10	10	10
glucose	50	50	50

Table 1. Composition of experimental solutions (mmol l^{-1}) (Nicolson, 1992). The pH was adjusted to 7.00 by adding 1 N HCl. Osmolality of all solutions was 390 mOsm/kg. Different K^+ concentrations were obtained by mixing solutions A and B.

Fluid secretion experiments

Malpighian tubules from larval *Tenebrio* were isolated as described by Wiehart et al. (2002). These were the free segments of the tubules, severed near the midgut and the rectal complex. Droplets of physiological saline (50µl) were placed in a Petri dish coated with Sylgard (10:1 base to curing agent) and covered with water saturated liquid paraffin. Two tubules were placed into each saline drop. The two ends of each tubule were pulled out of the bathing fluid and wrapped around Minuten pins, where they continued to secrete and the urine collected as discrete droplets in the liquid paraffin. Secreted drops were removed with a fine glass pipette and their diameters measured with a calibrated eyepiece graticule. The volume, and therefore the rate of secretion, was determined assuming the droplets to be spherical. The tubules were allowed to equilibrate for 20 min before three control readings were made at 15 min intervals. The bathing solution was then replaced with the experimental solution containing a high or low K^+ concentration and/or the test substances. Measurements were then taken over a 45-60 min period. Rates of secretion were expressed as a percentage of the third control rate reading.

Measurement of basolateral (V_{bl}) and transepithelial (V_{te}) potentials

Immediately after dissection a portion of a Malpigian tubule (3-5 mm) was suspended in a Ringer bath between two holding pipettes. The peritubular bath (300 µl) was perfused with Ringer solution at a rate of 1 ml/min. Intracellular (V_{bl}) and transepithelial (V_{te}) potentials were measured with 3 M KCl-filled microelectrodes (Borosilicate filament glass, Harvard; OD 1.2 mm, ID 0.69 mm; tip diameter < 0.5 µm; resistance 20-40 MΩ), connected to a Micro Probe System M-707 electrometer *via* Ag/AgCl wire. The reference electrode was a coarse, low resistance glass electrode (1 MΩ) filled with 3 M KCl/agar (2%) connected to earth *via* a Ag/AgCl wire. Cell impalement was accepted if a sudden drop in potential occurred; if the potential was stable for at least a few minutes; and if the electrode potential differed by not more than 3 mV from the baseline after withdrawal. Transepithelial potential was measured by advancing the microelectrode through the cell layer into the lumen of the tubule. The apical membrane potential (V_{ap}) was calculated as the difference between the measured transepithelial and basolateral potentials.

Statistics

Results are presented as mean values ± S.E.M. with the number of tubules (t) or number of measurements (n) in parentheses. The statistical significance of differences in fluid secretion or electrode potentials was evaluated by paired or unpaired Student's t-tests (two-tailed). A value of $P < 0.05$ was accepted as statistically significant.

Results

Basolateral and transepithelial potential differences under control conditions

Under control conditions tubules secreted spontaneously at a rate of 3.79 ± 0.6 nl/min (t = 72; measured within the first hour after dissection). Fig. 1 shows the frequency distribution of V_{bl} and V_{te} recorded during a large number of impalements of tubules in control Ringer (50 mmol l^{-1} K^+). The mean V_{bl} was -24 ± 0.43 mV (n = 166), with reference to the bathing medium, and the mean V_{te} was 24 ± 1.44 mV (n = 72), lumen positive. The apical membrane potential (V_{ap}) was not measured directly, but was derived by subtracting the mean V_{bl} from the mean V_{te}. Therefore V_{ap} in control Ringer was 48 mV, cell negative, with reference to the lumen.

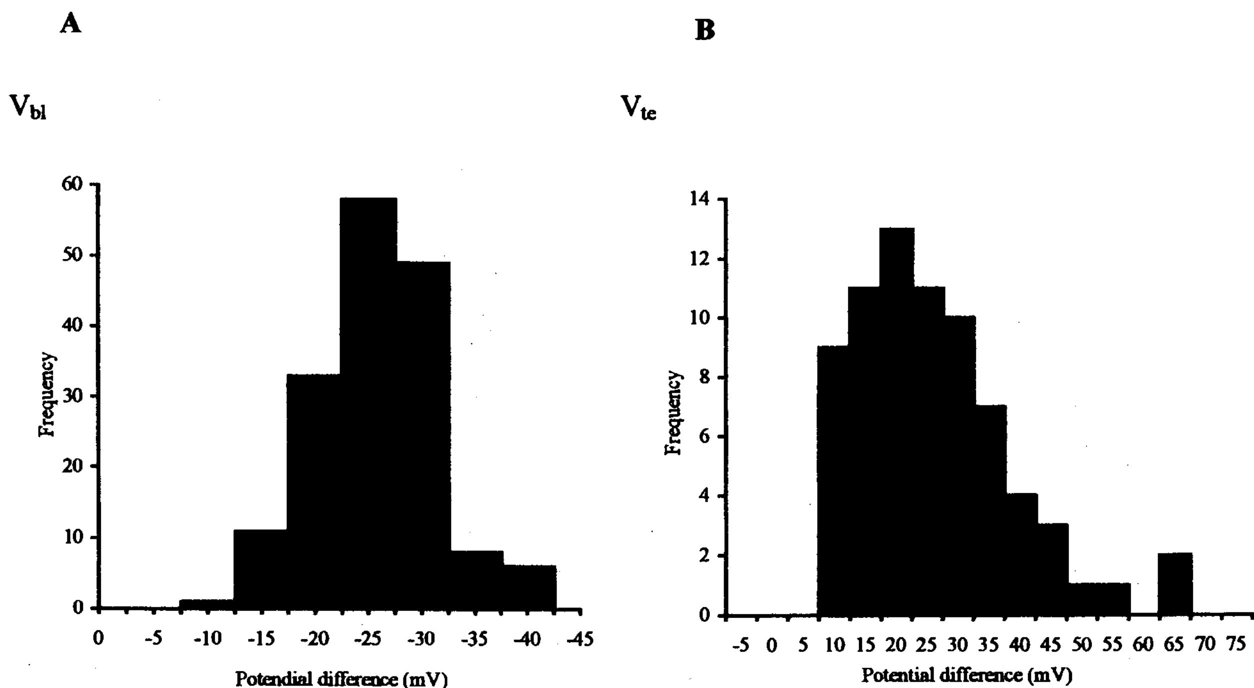

Fig. 1. Frequency distribution of (A) V_{bl} and (B) V_{te} measured in control Ringer (50 mmol l^{-1} K^+). Frequency is given for 5 mV intervals. Mean values were –24 ± 0.43 mV for V_{bl} (n = 166) and 24 ± 1.44 mV for V_{te} (n = 72).

The effect of changing external $[K^+]$ on fluid secretion

Fig. 2 summarizes the change in fluid secretion rates observed after changing $[K^+]$ and $[Na^+]$ in the bath. An increase of $[K^+]$ from 50 to140 mmol l^{-1} (Na^+ replaced by K^+), increased the fluid secretion rate from 5.38 ± 0.58 nl/min to 7.64 ± 0.60 nl/min ($P < 0.02$) (t = 8). Replacing the high $[K^+]$ Ringer with control Ringer reversed this effect. With a 10-fold drop in K^+ concentration (K^+ replaced by Na^+), secretion rates dropped from 6.38 ± 0.95 nl/min to 1.48 ± 0.52 nl/min ($P = 0.01$) (t = 8). Fluid secretion rates did not recover after the low K^+ medium was replaced with control Ringer and some tubules stopped secreting altogether, illustrating the importance of K^+ to the normal secretion of *Tenebrio* Malpighian tubules.

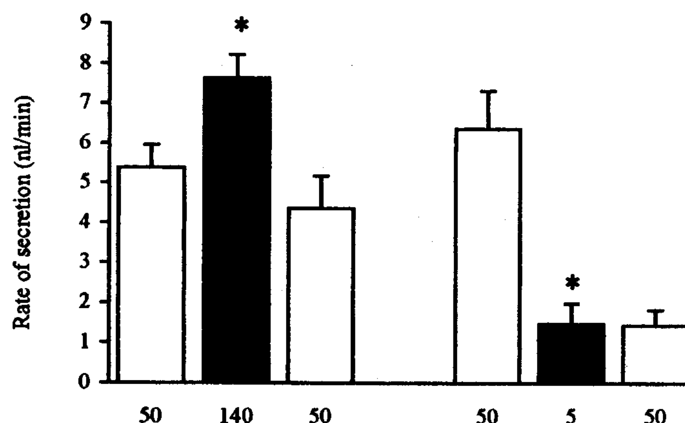

Fig. 2. Fluid secretion rates by *Tenebrio* tubules in response to different bath K^+ concentrations. A ten-fold decrease in the K^+ concentration irreversibly decreased fluid secretion rates ($P = 0.01$). Increasing the K^+ concentration to 140 mmol l^{-1} K^+ stimulated fluid secretion rates significantly ($P < 0.02$). Data are presented as means + 1 S.E.M (n = 8 for both secretion assays).

The effect of $[K^+]$ on membrane potentials

Fig. 3A shows the response of V_{bl} to varying K^+ concentrations. The microelectrode was then advanced into the lumen and the effect of the same series of K^+ concentrations was measured on V_{te}. The basolateral membrane was clearly sensitive to the bath $[K^+]$. It depolarized from -24 mV in control Ringer (50 mmol l^{-1} K^+) to –9 mV in the presence of a high K^+ concentration (140 mmol l^{-1}) and hyperpolarized to around -50 mV in 5 mmol l^{-1} K^+. The response of V_{bl} to a change in bath $[K^+]$ was immediate and is consistent with the presence of a significant K^+ conductance in the basolateral membrane of the tubule cells with the change in V_{bl} being 28 mV/decade (Fig. 3B).

Transepithelially there was an increase in potential as the K^+ concentration increased from 5 mmol l^{-1} K^+ to 140 mmol l^{-1} (Fig. 3A). Figure 4 summarizes the results. From the electrical potential profile presented in this figure, it is clear that there is a correlation between V_{ap} and V_{bl}.The overall result of increasing bath [K^+] from 5 mmol l^{-1} to 140 mmol l^{-1} was depolarisation of both V_{bl} and $V_{ap,}$ the change in V_{ap} being 60-77% of the change observed in V_{bl}.

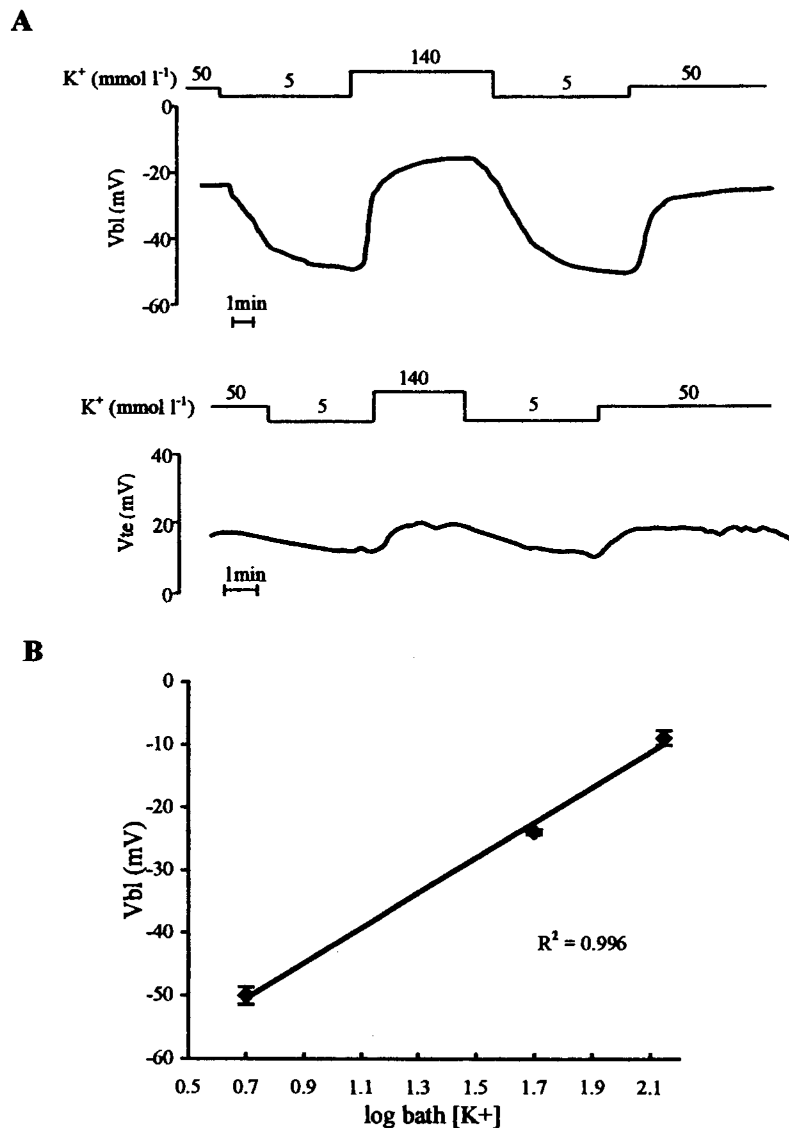

Fig. 3. The effect of changing the external K^+ concentration on V_{bl} and V_{te} in a paired experiment. V_{bl} hyperpolarizes in a low K^+ concentration and depolarizes in a high K^+ concentration. V_{te} hyperpolarizes as the K^+ concentration increases (A). V_{bl} as a function of log bath [K^+] shows a slope of the curve of 28 mV/decade (B). Each point shows the mean values ± 1 S.E.M of V_{bl}.

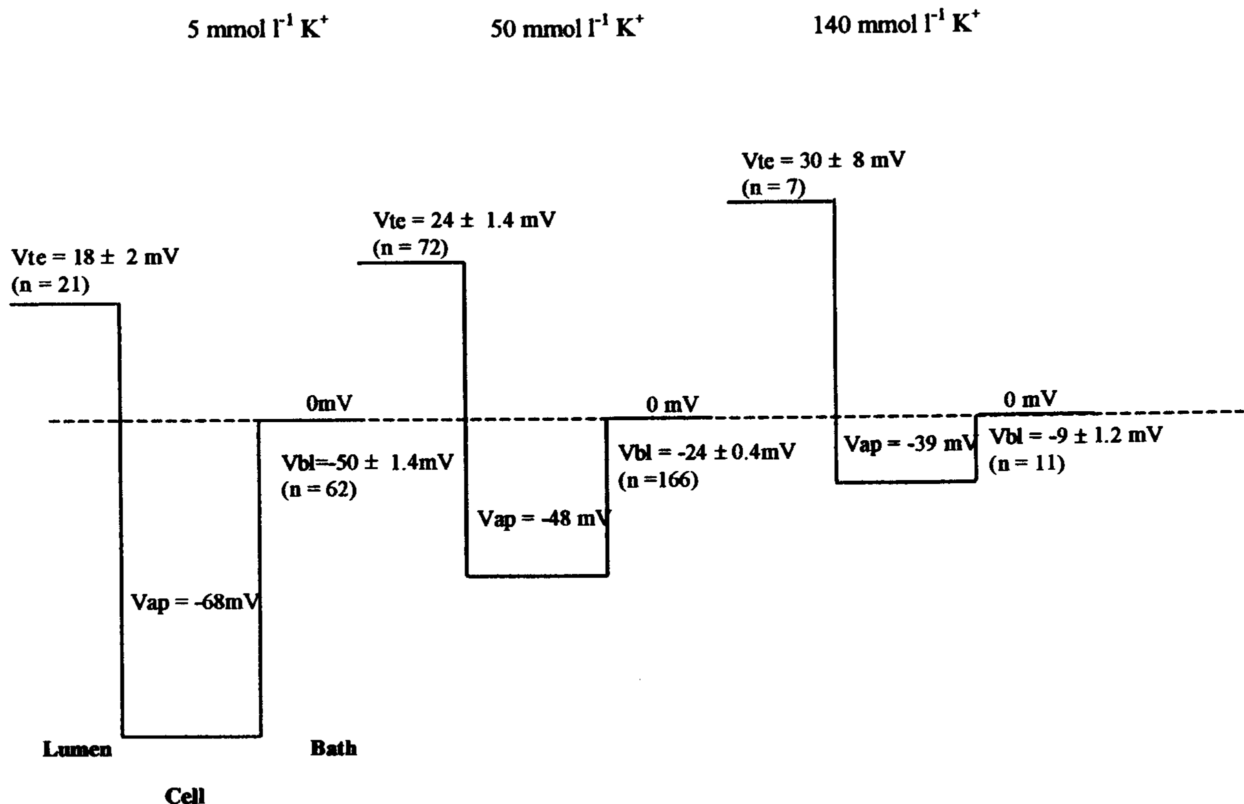

Fig. 4. Summary of the potential profiles across *Tenebrio* Malpigian tubules, measured in three different K^+ concentrations, mostly during unpaired experiments

The effect of barium

The presence of K^+ channels in the basolateral membrane of *Tenebrio* tubules and their relative importance in fluid secretion were investigated by the addition of 6 mmol l^{-1} Ba^{2+}, a known K^+ channel blocker, to the control bathing solution (50 mmol l^{-1} K^+). Fluid secretion rates decreased significantly from 4.17 ± 0.88 to 0.76 ± 0.24 nl/min within 45 min in the presence of Ba^{2+} (Fig. 5A; t = 8; $P < 0.004$). This drop in secretion rate was immediate, but the return of secretion rates to control levels after washout of Ba^{2+} took approximately 45 min.

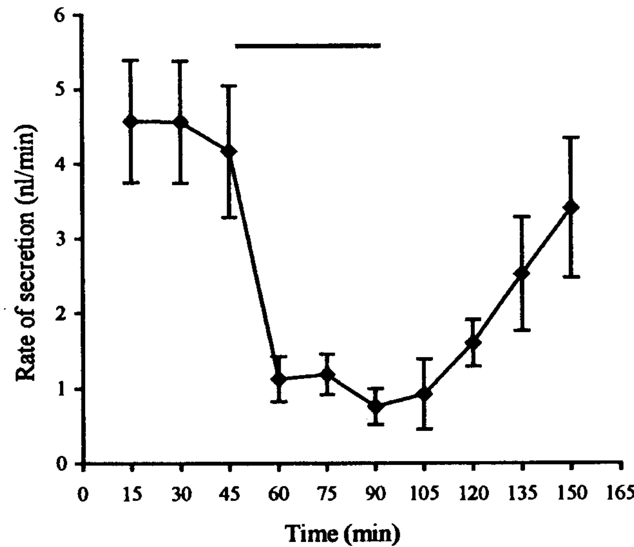

Fig. 5. The effect of 6 mmol l^{-1} barium on the fluid secretion rate of isolated Malpighian tubules (mean ± 1 S.E.M., n = 8 tubules). The horizontal bar indicates the time of exposure to barium.

Fig. 6 shows a typical response of V_{bl} to Ba^{2+}. Adding 6 mmol l^{-1} Ba^{2+} to control bath Ringer caused a hyperpolarization of the basolateral membrane from –24 ± 0.4 mV to –52 ± 1.9 mV and a slight decrease of V_{te} (not shown) from 24 ± 1.4 mV to 21 ± 1.9 mV (n = 47 and 34 respectively). The hyperpolarization of V_{bl} was sudden in some cells and more sluggish in others; the mean time being 4.6 ± 0.4 min (n=47). The effect of Ba^{2+} on the potential profile was completely reversible after washout for 5 to 8 min, with V_{bl} and V_{te} not significantly different from the initial control potentials. The addition of 6 mmol l^{-1} Ba^{2+} to 140 mmol l^{-1} K^+ Ringer hyperpolarized V_{bl} significantly from -9 ± 1.2 mV to -40 ± 3.6 mV over a period of 11.6 ± 2.2 min and caused a small but non-significant decrease in the V_{te} from 30 ± 7 mV to 27 ± 7.8 mV (n = 7 and 5 respectively; results not shown).

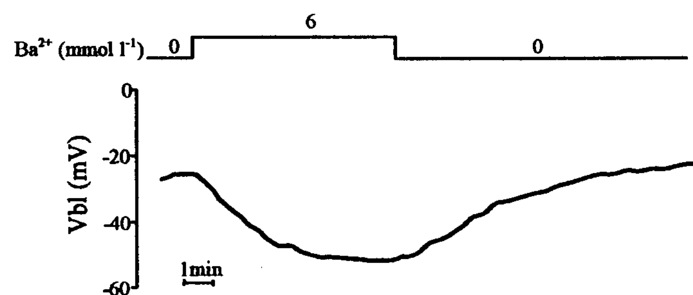

Fig. 6. A typical experiment illustrating the reversible effect of barium on the basolateral membrane potential.

Two different responses were seen when 6 mmol l^{-1} Ba^{2+} was added to a low bath [K^+] (5 mmol l^{-1}). In 8 of the 21 impalements the V_{bl} hyperpolarized significantly from a mean value of –52 ± 2 mV to –62 ± 3 mV ($P < 0.01$), while in the remaining 13 impalements a significant depolarisation from –60 ± 2 mV to –46 ± 3 mV was seen ($P < 0.001$). Fig. 7 illustrates both these responses. Possible reasons for this will be discussed later. The changes in the electrical profiles of Malpighian tubule cells bathed in the different K^+ concentrations in the presence of Ba^{2+} are summarized in Fig. 8.

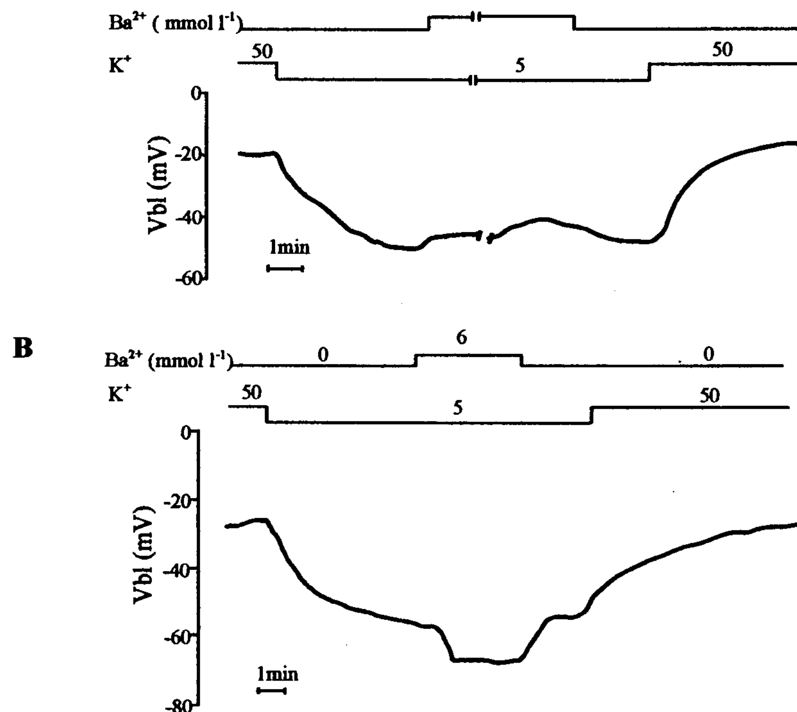

Fig. 7. Two different responses of V_{bl} to barium in the presence of a low K^+ concentration. (A) Significant depolarisation of the basolateral membrane potential was seen in 13 impalements ($P < 0.001$) and (B) a hyperpolarization was seen in 8 impalements ($P < 0.01$). Break in line due to disturbances. Time elapsed is 3 min.

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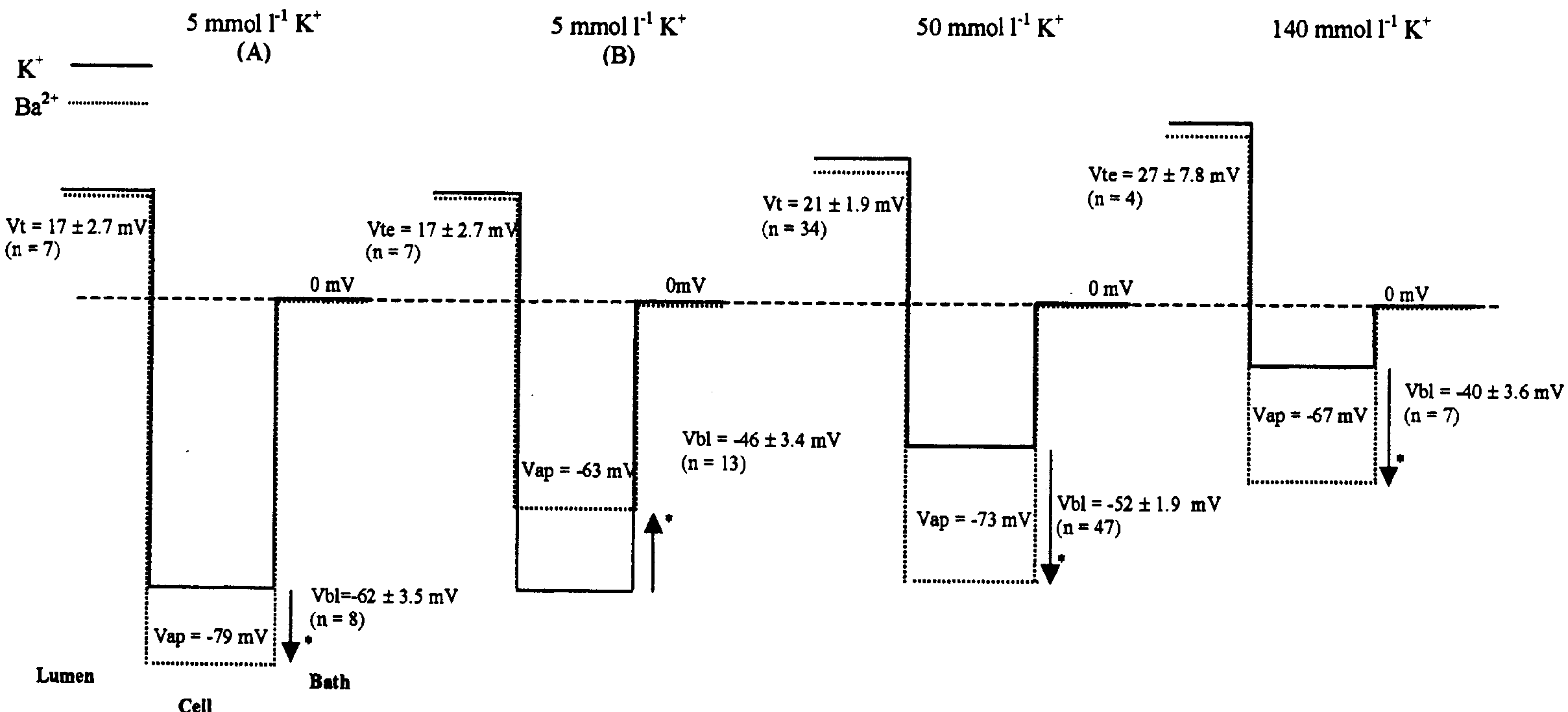

Fig. 8. Summary of the changes in V_{bl} and V_{te} in response to addition of 6 mmol l^{-1} barium observed in different bath K^+ concentrations. The solid line indicates the normal potential profile and the dotted line indicates the potential profile in the presence of barium. The asterisk indicates significant differences between mostly unpaired experiments.

The effect of rubidium on the basolateral membrane potential

To further investigate the nature of the potassium conductance of the basolateral membrane, K^+ ions were replaced by rubidium, a commonly used 'permeant' substitute for potassium. However, replacing 40 mmol l^{-1} K^+ of a control Ringer solution (50 mmol l^{-1} K^+) by 40 mmol l^{-1} rubidium caused a hyperpolarization of the V_{bl} of 35 mV (results not shown). The addition of 6 mmol l^{-1} rubidium to control Ringer had no effect on V_{bl} (n = 4). Clearly rubidium is not able to substitute for potassium in the Malpighian tubules of *Tenebrio*.

The effect of bumetanide on V_{bl} and V_{te}

The possibility of K^+ entering the cell through the $Na^+/K^+/2Cl$ cotransporter was investigated by means of the loop diuretic, bumetanide. Usually a concentration of 10 μmol l^{-1} is sufficient to block the $Na^+/K^+/2Cl$ co-transporter and higher concentrations are needed to block the K^+/Cl^- co-transporter (Palfrey and O'Donnell 1992). Bumetanide (10 μmol l^{-1}) significantly decreased fluid secretion rates of unstimulated tubules from 2.08 ± 0.22 to 0.55 ± 0.09 nl/min ($P < 0.01$; n =14). This inhibitory effect was reversible and after a wash out period of 45 min, fluid secretion rates had recovered to 1.56 ± 0.16 nl/min (Fig. 9). The effect of bumetanide on V_{bl} and V_{te} was investigated in the presence and absence of Ba^{2+}. None of the bumetanide treatments showed any significant effect on V_{bl} or V_{te} (n=5 and 7 respectively).

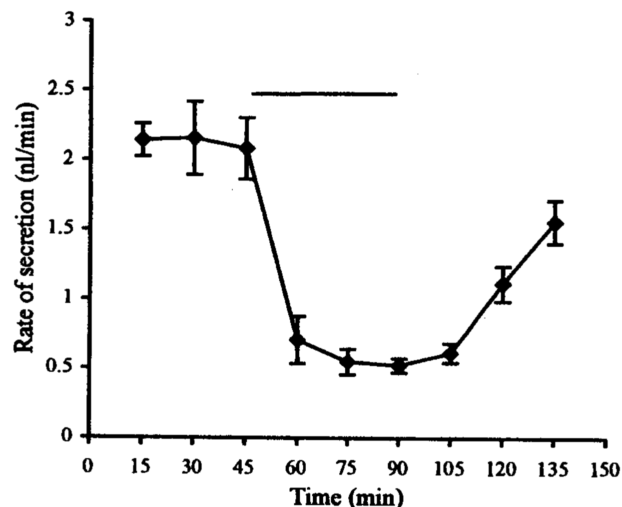

Fig. 9. The effect of 10 μmol l^{-1} bumetanide on the secretion rates of *Tenebrio* tubules (mean ± 1 S.E.M., n = 14 tubules). The horizontal bar indicates the time of exposure to bumetanide.

The effect of ouabain on V_{bl} and fluid secretion

The contribution of the basolateral Na^+/K^+-ATPase to K^+ uptake was investigated by blocking this ATP-dependent pump with 1 mmol l^{-1} ouabain. K^+ ions and ouabain compete for the same binding sites (Baker and Willis, 1970); therefore a high bath $[K^+]$ would decrease the effect of ouabain. Because, as previously shown, a low $[K^+]$ (5 mmol l^{-1}) irreversibly inhibits fluid secretion rates, the secretion assay was carried out in control Ringer (50 mmol l^{-1} K^+). The secretion rates of unstimulated tubules decreased from 5.6 ± 0.93 nl/min to 3.0 ± 0.6 nl/min after 15 min in the presence of 1 mmol l^{-1} ouabain (Fig. 10). A further decrease to 2.26 ± 0.36 nl/min was observed after an additional 45 min (n = 8). The inhibitory effect of ouabain on tubule secretion rates was irreversible. Basolateral and transepitelial membrane potentials showed no visible changes 10 min after the addition of 1 mmol l^{-1} ouabain (n=5).

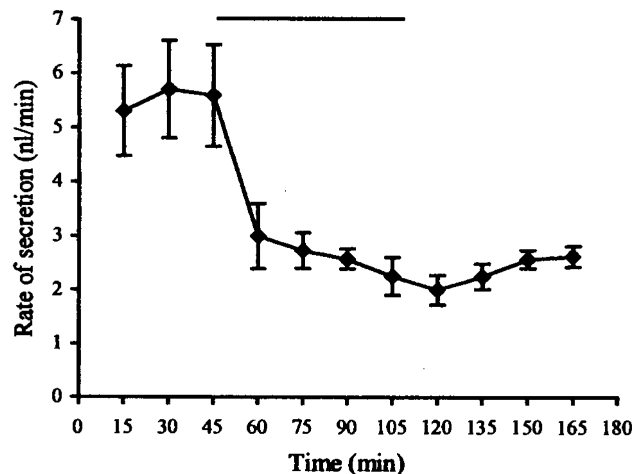

Fig. 10. The irreversible effect of 1 mmol l^{-1} ouabain on the secretion rates of *Tenebrio* tubules. Each point shows the mean ± 1 S.E.M. for 8 tubules ($P < 0.01$). The horizontal bar indicates the time of exposure to ouabain.

Discussion

Fluid secretion in Tenebrio tubules is controlled by bath $[K^+]$

We have shown that fluid secretion in Malpighian tubules of *Tenebrio* appears to be primarily dependent on the presence of K^+ in the bathing solution. In a low K^+ (5 mmol l^{-1}), high Na^+ solution secretion was greatly reduced and the effect was largely irreversible. Tubules of *Locusta migratoria* responded in a similar fashion (Anstee and Bell, 1975). An increase in bath $[K^+]$ significantly increased the rate of secretion

by *Tenebrio* Malpighian tubules, but a low Na^+ (6 mmol l^{-1}) solution (in the presence of a high K^+ concentration) did not affect fluid secretion. This has previously also been found in the forest ant *Formica polyctena* (Van Kerkhove et al., 1989) and the stick insect *Carausius morosus* (Pilcher, 1970). Haemolymph ion concentrations are very variable in insect species (see table 2, Van Kerkhove et al., 1989), and these ion substitution experiments may mirror physiological conditions.

Sodium dependent fluid secretion is primarily found in blood-sucking insects (e.g. *Rhodnius* and *Aedes*). These insects take huge, but infrequent blood meals and need to rid themselves as soon as possible of the high NaCl and water load, which renders them sluggish and easy prey. However, when not stimulated, tubules of *Rhodnius* secrete a K^+-rich fluid containing only low levels of sodium ions (Maddrell and O'Donnell 1992).

K^+ dependence of the basolateral membrane potential

The potential profile in Malpighian tubules of *Tenebrio* under control conditions is: V_{bl} -24 mV, with reference to the bath, and V_{te} 24 mV, lumen positive. The result is a mean V_{ap} of 48 mV, cell interior negative. This potential profile closely resembles that described for tubules of *Onymacris plana*, another tenebrionid beetle (Nicolson and Isaacson, 1987).

The basolateral membrane potential was very responsive to the K^+ concentration in the bath. Decreasing bath $[K^+]$ caused an immediate hyperpolarization, which was followed by an instant depolarization when the K^+ concentration was again increased. Although V_{bl} recovered, fluid secretion of tubules previously treated with a low $[K^+]$ did not (Fig. 2). A possible reason for this could be cell shrinking, but there is no microscopical evidence for this. It must be remembered that V_{bl} only measures the electrical effects of a low $[K^+]$ at the level of the basolateral membrane, but for fluid secretion in general a low $[K^+]$ could affect numerous transport mechanisms. Lack of availability of K^+ ions could slow down the putative apical V-ATPase, which would in turn affect the performance of the putative apical cation/nH antiporter. During fluid secretion, fluid is secreted over the entire tubule length and it would therefore take a longer period of time for the mechanisms to recover and produce enough fluid within the tubule lumen to actually be measurable.

Basal K^+ permeability is high in Malpighian tubule cells of most insect species studied so far (see Nicolson 1993 for review) and only the tubule cells of *Aedes aegypti* show a detectable Na^+ permeability, which is further increased in the presence of cAMP (Sawyer and Beyenbach, 1985).

When V_{bl} was plotted as a function of log $[K^+]$, a linear function was found with a slope of 28 mV/decade (Fig 3B), and not 58 mV, as expected if K^+ alone determined the membrane potential. Either other ions play a role and/or the intracellular K^+ concentration drops in low $[K^+]$. If other ions had some importance (e.g. Na^+) the dependence of V_{bl} to log $[K^+]$ is expected to level off at lower $[K^+]$. This is not the case. A drop in intracellular $[K^+]$ seems more likely. Leyssens et al. (1993) have shown that the intracellular $[K^+]$ in tubule cells of *Formica* can drop to 75% of the initial value when the $[K^+]$ in the bath is decreased ten-fold. In *Tenebrio* cells a changing intracellular K^+ concentration dependent on the bath K^+ concentration thus seems feasible, but this will have to be confirmed by ion-selective microelectrode measurements. On the other hand, the intracellular $[K^+]$ in Malpighian tubule cells of *Locusta* seemed to remain fairly constant and was not affected by the bath $[K^+]$ (Baldrick et al., 1988).

Furthermore changing the bath K^+ concentration indicated that V_{ap} closely followed V_{bl} (60-77% of the change observed in V_{bl}). This has also been reported for tubules of *Formica* (Leyssens et al., 1993). Weltens et al, (1992) have measured the resistance of the apical and basolateral membranes in *Formica* tubule cells and found that the resistance ratio of the apical over the basolateral membrane is high. From the electrochemical model of an epithelium it can be understood that the circular current, caused by the different electromotive forces across the basolateral and apical membrane and across the paracellular shunt, will contribute to the actually measured basolateral and apical voltage differences (Weltens et al, 1992). The effect will be small in the membrane with the lowest resistance and high in the membrane with the relative higher resistance. The basolateral membrane is dominated by a high K^+ conductance (low resistance) so that the circular current will have little impact on the measured potential and V_{bl} approaches the equilibrium potential for K^+. The apical membrane on the other hand will be influenced appreciably by a change in

electromotive force (and thus of circular current) and 'follow' this change. This mechanism may also be operative in the tubules of *Tenebrio*.

The effect of barium on secretion rate and basolateral membrane potential

Barium is a frequently used K^+ channel blocker and has been shown to reduce K^+ movement across the basolateral membrane in Malpighian tubule cells of *Formica* (Weltens et al., 1992); *Rhodnius* (lower tubule) (Haley and O'Donnell 1997); *Drosophila* (O'Donnell et al., 1996); Aedes (Masia et al., 2000) and *Locusta* (Hyde et al., 2001). Applying barium will increase the basolateral resistance and therefore make the effect of the epithelial circular current more 'visible' on this membrane, as explained in the previous section. The electromotive force of the apical V-ATPase, sends a positive current into the lumen making the cell interior more negative. In control K^+ the electrochemical gradient for seems to be cell inward and in the presence of barium it becomes more difficult for K^+ to cross the basolateral membrane and to compensate for this loss of positive ions from the cell. Also the inward current across this larger resistance will cause a bigger potential drop across the membrane, hyperpolarizing it. This was substantiated by the study of Weltens et al. (1992) where the authors have shown that the hyperpolarization of the basolateral membrane in the presence of Ba^{2+} was abolished by bafilomycin A_1, an inhibitor of the V-ATPase.

In the present study Ba^{2+} reversibly reduced fluid secretion by 83%. In control saline basolateral and apical membranes responded by a marked hyperpolarization and V_{te} decreased slightly. This is most probably due to (1) blocking of the K^+ channels in the basolateral membrane and (2) the increase in electrical potential difference created across the apical membrane possibly by a putative proton pump (V-ATPase) and (3) increase in the basolateral resistance and in the hyperpolarizing effect of the circular current as explained above.

The hyperpolarization of the basolateral membrane in some cells of *Tenebrio* tubules was notably slower than in others. This could indicate impeded access of Ba^{2+} to its site of interaction, possibly along the lateral spaces.

Different K^+ channel types are mostly classified according to their single channel conductance or ligands, rather than by their gating kinetics. The basolateral barium-

sensitive K^+ channels found in *Tenebrio* tubule cells appear to be specific for K^+ ions and are impermeable to rubidium. This became evident when the equimolar substitution of K^+ by rubidium caused a hyperpolarization of the basolateral membrane potential, similar to the hyperpolarization found in the presence of a low $[K^+]$. If rubidium were able to substitute for K^+, a slight depolarization of the basolateral membrane potential would have been expected with the addition of 6 mmol l^{-1} rubidium to control Ringer, as this would have been sensed as an increase in total K^+ concentration, but no effect was seen. In comparable studies, substitution of K^+ ions with rubidium caused a 50% decrease in fluid secretion of *Locusta* tubules and a hyperpolarization of V_{bl} (Hyde et al., 2001; Pivovarova et. al., 1994b). This is in contrast to K^+ channels found in the midgut of *Manduca* where rubidium substituted for K^+ ions to a greater extent (Schirmanns and Zeiske, 1994); and in the Malpighian tubules of the black field cricket, *Teleogyllus oceanicus*, a concentration of 8.6 mmol l^{-1} rubidium caused a 10% increase in fluid secretion rates with rubidium almost completely replacing K^+ in the secreted fluid (Marshall and Xu, 1999). In *Locusta* tubule cells, an increase in intracellular rubidium was seen when K^+ was replaced by rubidium, but the rubidium was not transferred to the lumen via the apical K^+/H^+ exchanger, indicating that the selectivity to K^+ ions does not lie within the K^+ channel, but rather the putative apical K^+/H^+ exchanger, responsible for transporting K^+ to the lumen (Pivovarova et. al., 1994b). In contrast to the K^+/H^+ exchanger of *Teleogyllus* tubule cells, this exchanger in *Locusta* cells appears to have a much higher affinity for K^+ ions than for rubidium. Whether this is the same for tubule cells of *Tenebrio* has yet to be determined.

The effect of barium in low K^+ concentrations

In the presence of a low bath $[K^+]$ (5 mmol l^{-1}), Ba^{2+} either hyperpolarized or depolarized V_{bl}. Providing that V_{bl} follows the Nernst potential for K^+, for a V_{bl} of -52 mV, K^+ would be at equilibrium if the intracellular $[K^+]$ were 40 mmol l^{-1}. This value is close to that found by Leyssens et al., (1993) in tubule cells of *Formica*. Any intracellular concentration below 40 mmol l^{-1} K^+ would cause K^+ to move into the cell. Blocking this inward movement with barium would result in a hyperpolarization (see Weltens et al. 1992). Cells that show a depolarization of the basolateral membrane in the presence of barium most probably have an outward electrochemical gradient for K^+, the favorable electrochemical gradient created by the apical proton

pump being too low to draw K^+ ions into the cell. The addition of barium blocks the K^+ ions from leaving the cell via the K^+ channels and results in a depolarization of V_{bl}. This has also been found in the lower tubules of *Rhodnius* (Haley and O'Donnell, 1997), which are responsible for ion reabsorption, and in the upper secreting tubule of the same insect (Ianowski et al. 2002). Secretion by the upper Malpighian tubules of *Rhodnius* is not inhibited by Ba^{2+}, because it is driven by Na^+ rather than K^+ (Ianowski et al. 2002). Clearly at physiological (50 mmol l^{-1}) and higher bath K^+ concentrations, which we have shown to increase fluid secretion, the hyperpolarization of the basolateral membrane indicates that the net movement of K^+ ions is from the bath into the cell.

The effect of bumetanide

Basolateral entry of ions via the $Na^+/K^+/2Cl$ cotransporter has been implicated in Malpighian tubules of other insect species. In the present study we tested the effect of bumetanide, which blocks this cotransporter, on fluid secretion rates of *Tenebrio* Malpighian tubules and found strong inhibition in non-stimulated tubules. Bumetanide also inhibits fluid secretion in cyclic AMP stimulated tubules of *Rhodnius* (O'Donnell and Maddrell, 1984; Ianowski et al., 2002) and *Aedes* (Hegarty et al., 1991); stimulated and unstimulated tubules of *Drosophila* (Linton and O'Donnell, 1999); and unstimulated tubules of *Locusta* (Baldrick et al., 1988) and *Formica* (Leyssens et al., 1994). However, due to the relatively high concentration of bumetanide required to partially inhibit fluid secretion in *Drosophila*, Linton and O'Donnell (1999) suggested that bumetanide inhibited a K^+/Cl^- cotransporter rather than a $Na^+/K^+/2Cl$ cotransporter. According to the results of the fluid secretion assay, it seems highly likely that K^+ ions cross the basolateral membrane of *Tenebrio* tubules *via* the $Na^+/K^+/2Cl$ cotransporter, in addition to passage through channels.

Bumetanide affects electroneutral transport mechanisms and the lack of a visible effect on membrane potentials supports this characteristic. This result is in accordance with findings of previous studies on *Locusta* (Baldrick et al., 1988) and *Formica* (Leyssens et al., 1994).

The effect of ouabain

The role of a Na^+/K^+-ATPase in the Malpighian tubules of *Tenebrio* has been substantiated, given that ouabain, a specific inhibitor of the Na^+/K^+-ATPase, decreased fluid secretion irreversibly by 52%. Fluid secretion by unstimulated tubules of *Aedes* (Hegarty et al., 1991) and *Locusta* (Anstee and Bowler, 1979) is similarly affected in the presence of 1 mmol l^{-1} ouabain. In contrast, ouabain stimulates fluid secretion in tubules of *Rhodnius* (Maddrell and Overton, 1988) and *Drosophila* (Linton and O'Donnell, 1999). Inhibiting the Na^+/K^+-ATPase disrupts its active role of transporting three Na^+ ions from the cell interior to the outside and two K^+ ions from the outside into the cell (De Weer, 1992) resulting in an increase in intracellular Na^+ concentration (Maddrell and O'Donnell, 1992). In stimulated tubules of *Rhodnius*, when fluid secretion is predominantly driven by the presence of a high Na^+ concentration, the presence of ouabain increases the secretion rate as well as the Na^+ concentration in the secreted fluid (Maddrell and Overton, 1988). In the present study, ouabain had no effect on V_{bl}. However, in both *Rhodnius* and *Drosophila* V_{bl} depolarized in the presence of ouabain, indicative of an increase in intracellular Na^+ concentration and possibly a decrease in intracellular K^+ levels. Considering that the Na^+/K^+-ATPase provides a route of K^+ entry into the tubule cells, blocking this pump decreases fluid secretion in insects where K^+ is the main player in driving fluid secretion.

In this study we have shown that the basolateral K^+ uptake is an important factor determining fluid secretion rates of *Tenebrio* Malpighian tubules. K^+ ions are transported across the basolateral membrane via barium sensitive K^+channels, and via the electroneutral $Na^+/K^+/2Cl$ cotransporter and the Na^+/K^+-ATPase. The nature of the basolateral K^+ channels and the possible regulation of the various K^+ uptake mechanisms by endogenous factors are subjects for further investigation.

Financial support was provided by a bilateral award (Bil98/53) under the Flemish-South African agreement on scientific and technological cooperation, and by the South African National Research Foundation and the University of Pretoria.

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Paper 5

K^+ transport in Malpighian tubules of *Tenebrio molitor*: is a K_{ATP} channel involved?

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Journal of Experimental Biology (in press)

Contribution of co-authors other than supervisor(s)

P. Steels and G. Klein advised and spent many hours in discussions and brainstorming sessions.

Note: **Paper 5** is written in the first person for thesis purposes.

Abstract

The presence of ATP-regulated (K_{ATP}) K^+ channels in *Tenebrio molitor* Malpighian tubules was investigated by examining the effect of glibenclamide on both fluid secretion and basolateral membrane potentials (V_{bl}). Glibenclamide, a K_{ATP} channel blocker, slowed fluid secretion of *Tenebrio* tubules. In low bath K^+ concentration (5 mmol l^{-1}), glibenclamide either hyperpolarized or depolarized V_{bl}, resembling the effect seen with Ba^{2+} (Wiehart et al., 2003). Subsequent addition of 6 mmol l^{-1} Ba^{2+} caused a further hyper- or depolarization of V_{bl}. In control Ringer (50 mmol l^{-1} KCl, 90 mmol l^{-1} NaCl) glibenclamide had no visible effect on V_{bl}. The effect of ouabain was investigated in low bath [K^+] in the presence of Ba^{2+}. V_{bl} responded by a small but significant hyperpolarization from -51 ± 4 mV to -56 ± 4 mV ($n = 16$; $p<0.001$) in response to 1 mmol l^{-1} ouabain. Repeating the experiments in the presence of both glibenclamide and Ba^{2+} resulted in a depolarisation of V_{bl} when ouabain was added. In low bath K^+ (high Na^+) the Na^+/K^+-ATPase is expected to function at a high rate. In the presence of Ba^{2+}, replacing Na^+ by K^+ rapidly depolarized V_{bl}, but this was followed by a re-polarization. Repeating the experiments in the presence of glibenclamide markedly reduced the depolarizing effect and abolished the re-polarization, with a gradual decrease in the sensitivity of V_{bl} to the surrounding [K^+]. These results suggest the presence of K_{ATP} channels in the basolateral membrane. Glibenclamide had no visible effect on V_{bl} in high K^+ or in the absence of Ba^{2+}, indicating that other highly conductive K^+ channels may mask the effect on K_{ATP} channels. This is the first demonstration of the presence of K_{ATP} channels in an insect epithelium.

Key words: K^+ transport, KATP channel, Malpighian tubules, *Tenebrio molitor*, glibenclamide, basolateral membrane potential, fluid secretion rate.

Introduction

Insect Malpighian tubules play a pivotal role in maintaining ion and water homeostasis in the face of extreme and variable conditions. Electrophysiological studies indicate that in Malpighian tubules of *Tenebrio* (Wiehart et al., 2003), in common with other insect species (for reviews see Pannabecker, 1995; Van Kerkhove, 1994), the prime mover of primary urine production is active K^+ transport across the epithelium.

Basolateral entry of K^+ occurs mainly via Ba^{2+} sensitive K^+ channels (Nicolson and Isaacson, 1990; Leyssens et al., 1993) and the $Na^+/K^+/2Cl$ and K^+/Cl^- cotransporters. However, active K^+ transport via a basolaterally located Na^+/K^+-ATPase has been suggested for a number of insect species (Anstee and Bowler, 1979; Maddrell and Overton, 1988; Caruso-Neves and Lopes, 2000; Linton and O'Donnell, 1999).

In transporting epithelia of vertebrates the activity of the basolateral Na^+/K^+-ATPase is directly linked to the basolateral K^+ conductance (Grasset et al., 1983; Matsumura et al., 1984). Inhibition of the Na^+/K^+-ATPase by ouabain increases the intracellular ATP concentration, which in turn reduces the open probability of ATP-regulated K^+ (K_{ATP}) channels (Balaban et al., 1980; Hurst et al., 1993; Urbach et al., 1996).

In Na^+-reabsorbing epithelia, transport of Na^+ is facilitated by passive entry mechanisms in the apical membrane and an active Na^+-translocation step- the basolateral Na^+/K^+-ATPase. K_{ATP} channels recycle the obligatory influx of K^+ via the Na^+/K^+-ATPase (Mauerer et al., 1998; Wang et al., 1990). This recycling process prevents intracellular K^+ accumulation and maintains a favorable electrical gradient for Na^+ transport across the apical membrane (Hurst et al, 1993). Far less is known about the presence of K_{ATP} channels in K^+ secreting epithelia. Wang et al. (1990), however, have documented the presence of a low conductance K_{ATP} channel in the K^+ secreting principal cells of the rat cortical collecting tubule.

A role for K_{ATP} channels in insects is expected to be different. Secretion of K^+ from cell to lumen in insect Malpighian tubules is generally thought (see Nicolson, 1993) to occur *via* an apical cation/nH^+ antiporter. A vacuolar-type H^+-ATPase actively extrudes H^+ across the apical membrane, and this (1) energizes the antiporter, enabling exchange of protons for K^+ (or Na^+) and (2) keeps the cell at a negative potential, beyond the Nernst potential for K^+, thereby creating an inward electrochemical gradient for K^+ across the basolateral membrane (Leyssens et al., 1993; Wiehart et al., 2003). The possible function of K_{ATP} channels, if present, in Malpighian tubule cells may be to contribute to K^+ uptake in certain conditions, in parallel with the Na^+/K^+-ATPase and other K^+ uptake mechanisms.

K_{ATP} channels were first discovered in cardiac myocytes (Noma, 1983) and were later found in many other tissues (Ashcroft and Ashcroft, 1990). The properties of K_{ATP} channels have been described (for reviews see Ashcroft and Ashcroft, 1990; Seino, 1999; Wang et al., 1992). Depending on location these channels exhibit differences in function and therefore differ somewhat in their properties: however all K_{ATP} channels are highly selective for K^+ ions, displaying inward rectification with inward conductances ranging between 20-300 pS. They are regulated by the intracellular ATP concentration and blocked by the highly specific sulfonylureas, of which glibenclamide and tolbutamide are best described (Ashcroft and Ashcroft, 1990).

The present study investigates the possible presence of K_{ATP} channels in the tubule epithelium of *Tenebrio* by testing the effect of glibenclamide on Malpighian tubule secretion rates and basolateral membrane potentials. We investigate the possibility of a functional link between the activity of the basolateral Na^+/K^+-ATPase and K^+ conductance via the proposed K_{ATP} channels, by first stimulating this pump with an increase in Na^+ concentration and then inhibiting it by means of ouabain. Finally, we examine the basolateral membrane sensitivity to the bath K^+ in the presence and absence of glibenclamide. To our knowledge this is the first study that investigates the presence of K_{ATP} channels in the Malpighian tubules of an insect.

Material and Methods

Animals

Tenebrio larvae were kept under crowded conditions at room temperature (20-23°C) and fed on a diet of bran and apple. Care was taken in selecting mealworms of similar size for all experiments.

Artificial salines

The composition of the control bathing solution (in mmol l^{-1}) was as follows (Nicolson, 1992): NaCl 90, KCl 50, $MgCl_2$ 5, $CaCl_2$ 2, $NaHCO_3$ 6, NaH_2PO_4 4, glycine 10, proline 10, serine 10, histidine 10, glutamine 10, and glucose 50. The pH was adjusted to 7.0 with HCl and the osmolality was kept at 390 mOsm/kg. Low $[K^+]$ solutions were obtained by replacing KCl with NaCl, and low-Na solutions by replacing NaCl with KCl (low-Na^+ solutions contained 6 mmol l^{-1} Na^+). Solutions were freshly prepared each week, filtered through 0.22 µm Millipore filters and kept at 2 °C until used. The pH was measured daily before use. In low Na^+ experiments and experiments containing Ba^{2+}, NaH_2PO_4 was omitted from all salines to maintain constant osmolality and prevent precipitation. Control experiments in which NaH_2PO_4 was omitted showed no change in secretion rate or electrical profile.

The following pharmacological substances were tested on Malpighian tubule preparations: barium chloride (Sigma), ouabain (Fluka), glibenclamide (Sigma), cyclic AMP (Sigma).

Fluid secretion experiments

The technique of measuring fluid secretion rates was described previously (Wiehart et al., 2002). Secretion was measured in control Ringer's containing 1 mmol l^{-1} cyclic AMP (control), and subsequently in control Ringer's containing cyclic AMP and glibenclamide. Rates of secretion were expressed as a percentage of the third control rate reading. Six to ten replicates were done for each experiment.

Electrical potential difference measurements

This method was described in detail previously (Wiehart et al., 2003). In short, a portion of a Malpigian tubule (3-5 mm) was suspended in a Ringer bath. Intracellular (V_{bl}) measurements were performed with 3M KCl-filled microelectrodes. Cell impalement was accepted if a sudden drop in potential occurred; if the potential was stable for at least a few minutes; and if the electrode potential differed by not more than 3 mV from the baseline after withdrawal.

Statistics

Results are presented as mean values ± S.E.M. with the number of tubules (t) or number of measurements (n) in parentheses. The statistical significance of differences in fluid secretion or electrode potentials was evaluated by paired or unpaired Student's t-tests (two-tailed). A value of $P < 0.05$ was accepted as statistically significant.

Results

The effects of glibenclamide on fluid secretion

Glibenclamide, a sulfonylurea derivative known to block K_{ATP} channels, was tested on *Tenebrio* tubules. Application of either 0.1 or 0.5 mmol l^{-1} glibenclamide inhibited the fluid secretion rates by 34.2 ± 5.9% (n = 8) and 42.2 ± 6.6 % (n = 6) respectively, after 15 min (Fig. 1). The inhibitory effect of glibenclamide was not reversible after washout. Subsequent addition of the endogenous diuretic peptide, Tenmo-DH_{37} (100 nmol l^{-1}) however, increased fluid secretion rates indicating that tubules were still viable.

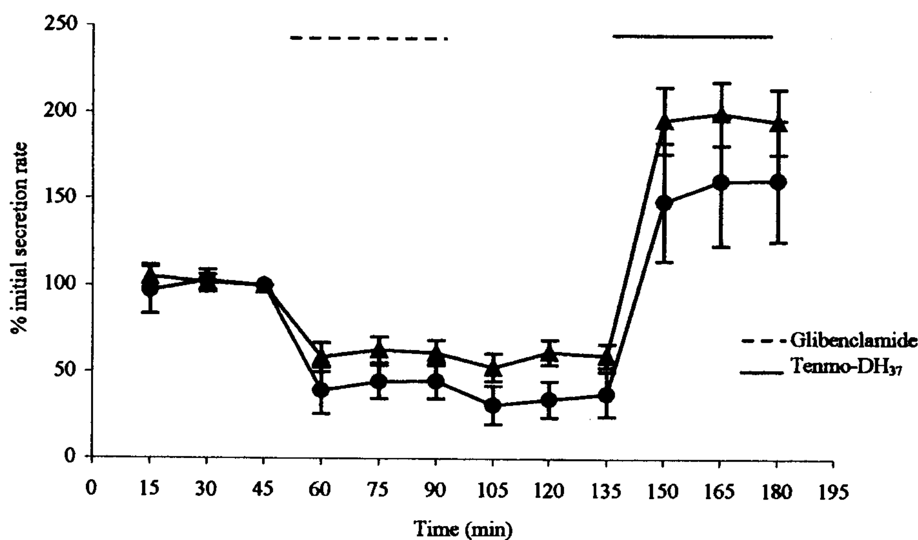


Fig. 1 Effect of glibenclamide on fluid secretion by tubules of *Tenebrio*. Glibenclamide was tested at 0.1 mmol l^{-1} (triangles) and 0.5 mmol l^{-1} (circles) in control Ringer (50 mmol l^{-1} K^{+}). Secretion rates recovered after stimulation with Tenmo-DH_{37} (100 nmol l^{-1}). The horizontal bars indicate the time of exposure to glibenclamide (broken bar) and to Tenmo-DH_{37} (solid bar). Data are presented as means ± 1 S.E.M. for 7-8 tubules.

The effect of glibencamide on V_{bl}

In a low bath $[K^+]$ (5 mmol l^{-1}) the addition of 0.5 mmol l^{-1} glibenclamide elicited a similar change in V_{bl}, to that previously seen in the presence of Ba^{2+} (Wiehart et al., 2003), although to a lesser degree. V_{bl} responded to glibenclamide by either a small but significant hyperpolarization from -56.6 ± 3.3 mV to -59.7 ± 3.3 (Fig. 2A; $P = 0.01$; n = 8) (in one experiment there was a marked hyperpolarization of 12 mV) or a significant depolarization (Fig. 2B) from –68.3 ± 3.8 mV to -52.3 ± 1.5 mV ($P = 0.008$; n = 4). Subsequent addition of Ba^{2+} reinforced either the hyperpolarization or the depolarization initiated by glibenclamide (both responses are shown in Fig. 2A and B).

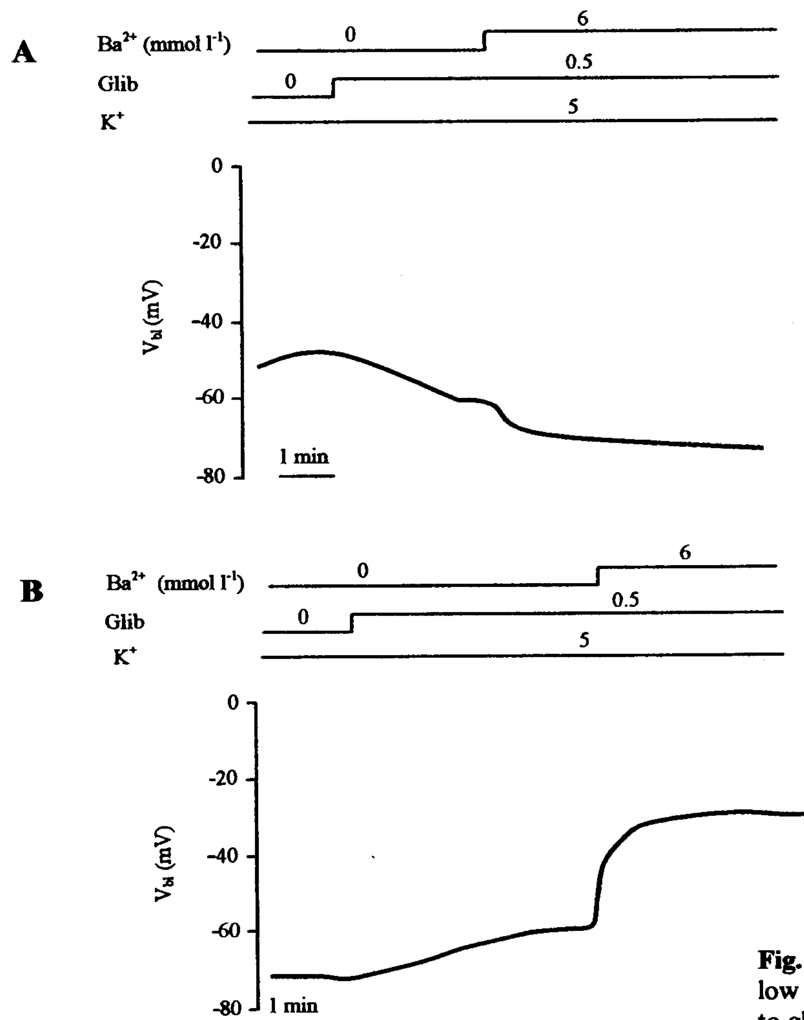


Fig. 2 Response of V_{bl} to glibenclamide. In a low K^+ bath (5 mmol l^{-1} K^+) V_{bl} responded to glibenclamide by either (A) a small but significant hyperpolarization of 3.6 ± 1.2 mV ($P = 0.01$; n = 8) or (B) a significant depolarization of 9 ± 1.5 mV ($P = 0.008$; n = 4). Addition of 6 mmol l^{-1} Ba^{2+} reinforced the initial response of glibenclamide.

The experimental protocol was reversed to determine whether glibenclamide had an effect on V_{bl} in the presence of Ba^{2+}. Again glibenclamide caused a further hyperpolarization of V_{bl} from -51.8 ± 5.6 mV to -54.7 ± 5.8 mV (n = 5; $P < 0.006$) or depolarization from -45 mV to -41 mV (n = 1) (results not shown), following the response initiated by Ba^{2+}. The addition of glibenclamide to control Ringer (50 mmol 1^{-1} K^{+}) had no visible effect on V_{bl} (n = 10).

V_{bl} in the presence of ouabain

Previously we found that ouabain (1 mmol 1^{-1}) added to control Ringer (50 mmol 1^{-1} K^{+}) significantly reduced fluid secretion, but had no visible effect on V_{bl} (Wiehart et al., 2003). Blocking of the outward electrogenic current of the Na^{+}/K^{+} pump by ouabain is expected to cause - if anything - a depolarization of the membrane. The absence of a visible effect could be due to the high conductance (mainly due to K^{+}) of the basolateral membrane. Ouabain had a variable effect on V_{bl} in low bath K^{+} (5 mmol l^{-1}), the tubule cells responding either by a small hyperpolarization of 3 mV (n = 1) or a depolarization of 3 to 6 mV (n = 3; results not shown). This variable result was further investigated in the presence of 6 mmol l^{-1} Ba^{2+} to reduce the impact of highly conductive K^{+} channels. Two of 16 experiments showed a slight depolarization of V_{bl} in the presence of ouabain. Surprisingly, in all other experiments, V_{bl} responded by a small, but significant hyperpolarization. Fig. 3A shows the result of an experiment in which V_{bl} hyperpolarized from -64 mV to -73 mV. The observed hyperpolarization occurred gradually over a period of 3-5 minutes and dropped back to pre-ouabain treated potentials within 1 min of washout. Fig. 3B summarizes the results of all 16 experiments. Ouabain had no detectable effect on V_{bl} in control Ringer (50 in mmol l^{-1} K^{+}) in the presence of Ba^{2+}(n = 4).

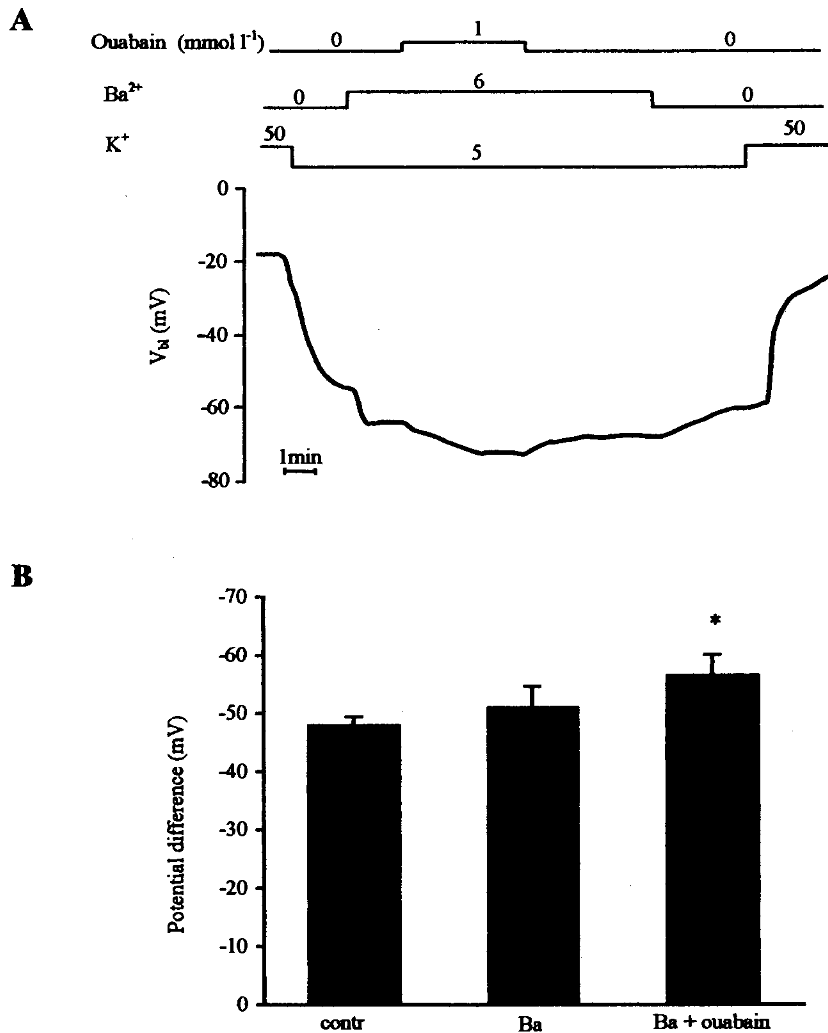


Fig. 3 (A) Effect of ouabain on V_{bl} in low bath K^+ (5 mmol l^{-1}) measured in the presence of Ba^{2+}. (B) Summary of the response of V_{bl} to ouabain. Data are presented as means ±1 S.E.M. (n = 16; $P<0.001$).

The effect of ouabain on V_{bl} in the presence of glibenclamide

The effect of ouabain was tested again, but this time in the presence of glibenclamide (and Ba^{2+}). The experiments in Fig. 4 illustrate the result. After the addition of glibenclamide and Ba^{2+}, which either caused a hyperpolarization (Fig 4A) or depolarisation (Fig 4B) of V_{bl}, the addition of 1 mmol l^{-1} ouabain always resulted in a depolarization of V_{bl}, averaging 8.5 ± 1.4 mV (n =8; P = 0.001).

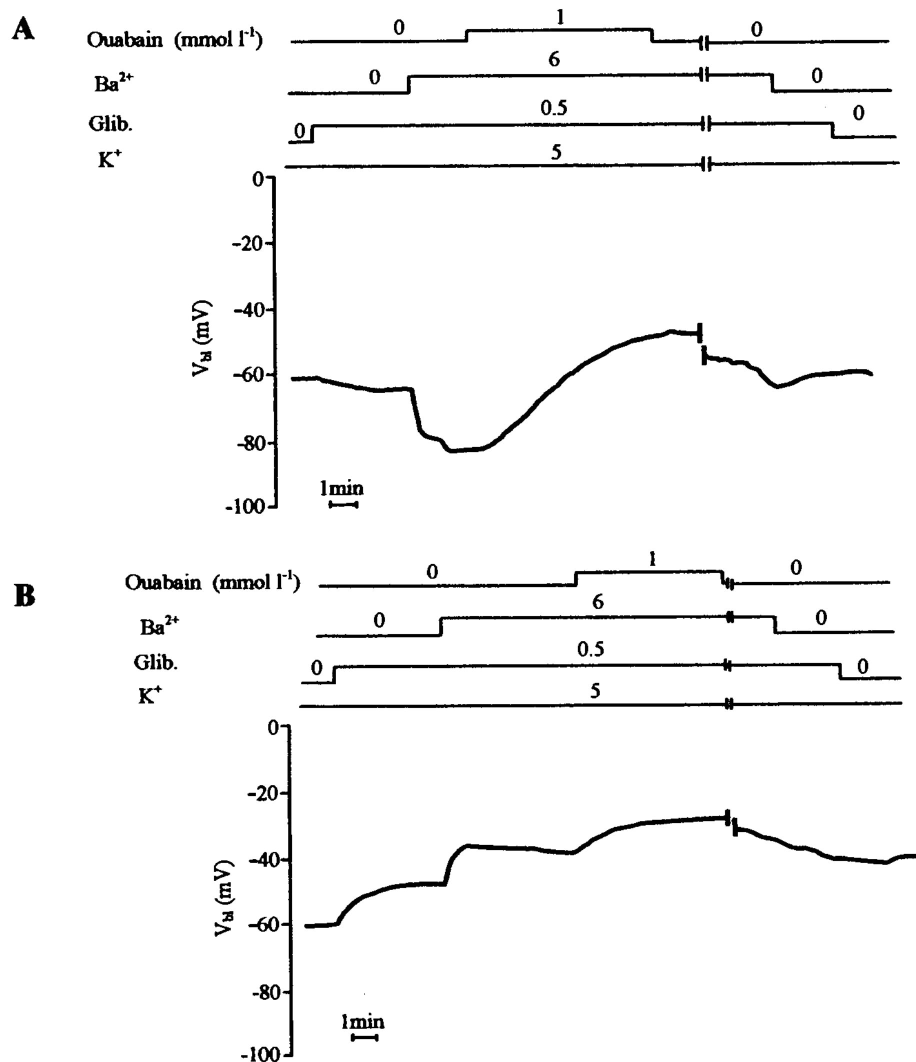


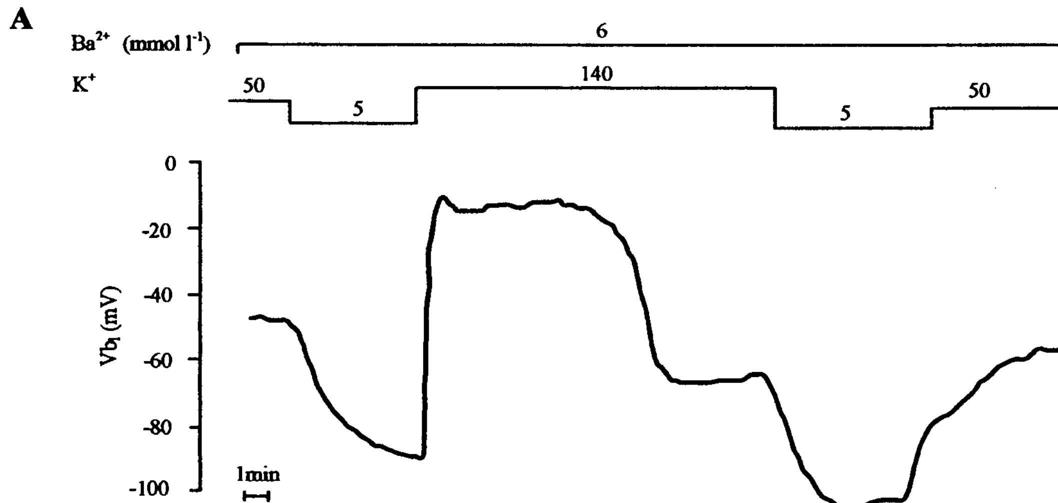
Fig. 4 Typical experiments showing the effect of ouabain on V_{bl} in the presence of glibenclamide and Ba^{2+}. The hyperpolarization (A) or depolarisation (B) of glibenclamide and Ba^{2+} is followed by a depolarisation of V_{bl} with the addition of 1 mmol l^{-1} ouabain. The experiments were carried out in low bath K^+ (5 mmol l^{-1}).

Further indications of K_{ATP} channels in the basolateral membrane

In the presence of Ba^{2+}, although a loss of K^+ sensitivity is expected, a change in the bath $[K^+]$ from 5 mmol l^{-1} K^+ to 140 mmol l^{-1} K^+ caused a depolarization of V_{bl} from -88.8 ± 2.7 mV to -13.7 ± 1.9 mV, followed by a re-polarization to –51.8 ± 7.0 mV beginning after 3 – 8 min (n = 6). A typical experiment is shown in Fig. 5A, and Fig.

5B summarizes the results of 6 experiments. Such a re-polarization was never seen after a 20 min period in a high [K^+] in the absence of Ba^{2+} (result not shown).

A



B

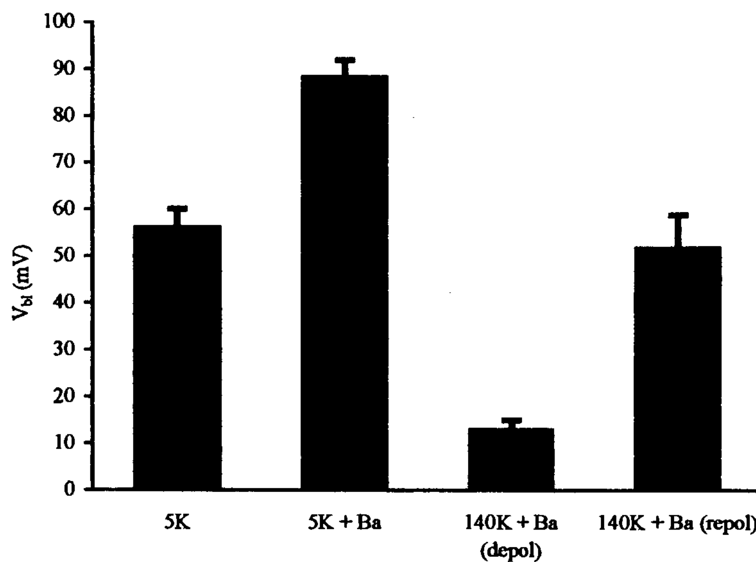


Fig. 5 (A) Response of V_{bl} to different bathing K^+ concentrations in the presence of Ba^{2+}. The depolarization of V_{bl} when changing the [K^+] from 5 mmol l^{-1} to 140 mmol l^{-1} is followed by a re-polarization after 3-8 min. (B) Summary of the effect of Ba^{2+} in various [K^+]. Data are presented as means ±1 S.E.M. (n = 6).

Fig. 6 shows the result of a similar experiment, but in the presence of 0.5 mmol l^{-1} glibenclamide. Although V_{bl} still depolarized by 43.1 ± 5.7 mV (n = 6) when the bath [K^+] was changed from a low (5 mmol l^{-1} K^+) to a high concentration (140 mmol l^{-1}

K^+), this was significantly less than the depolarization of 75 mV previously seen in the absence of glibenclamide. Furthermore no subsequent re-polarization of V_{bl} was seen in any of the experiments even after 30 min of high [K^+]. The rate of response of the basolateral membrane to either the high or low [K^+] was noticeably affected in the presence of glibenclamide. With both Ba^{2+} and glibenclamide present, V_{bl} hyperpolarized over a mean time of 15 min (n = 6) in response to low bath K^+, compared to 8 min (n = 7) in the presence of Ba^{2+} alone. Likewise V_{bl} depolarized over a mean period of 12 min (n = 6) in response to a high [K^+] compared to 3 min when only Ba^{2+} was present. During these experiments the basolateral membrane became increasingly less sensitive to the bath [K^+] with time. Reintroduction of a low bath [K^+] hyperpolarized V_{bl} to –57 ± 3.8 mV compared to the previous –66 ± 6.2 mV (n =6; P = 0.002).

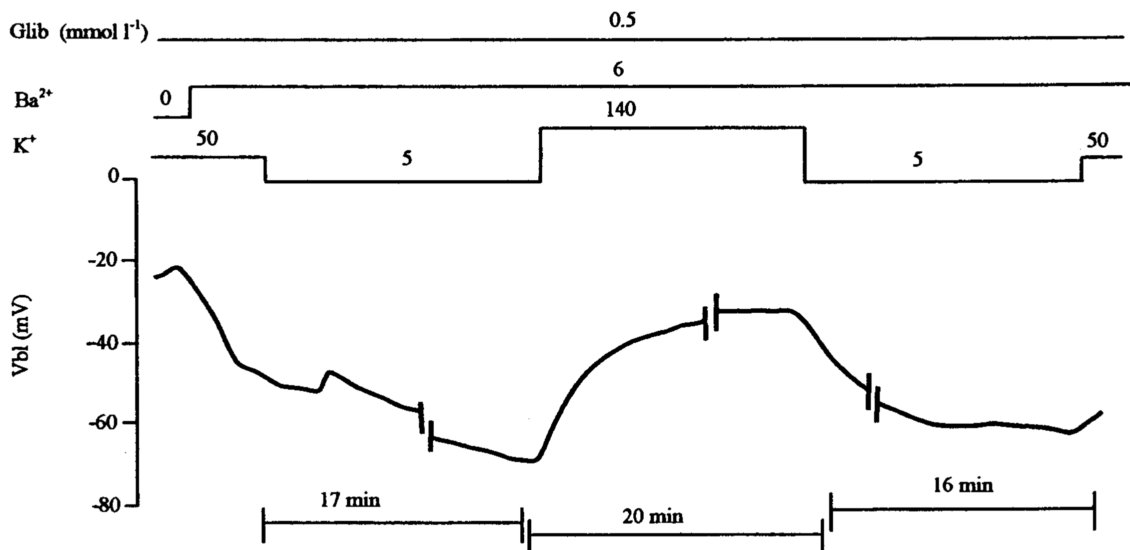


Fig. 6. Slow response of V_{bl} to different bathing K^+ concentrations in the presence of glibenclamide and Ba^{2+}. The depolarization of V_{bl} when changing the [K^+] from 5 mmol l^{-1} to 140 mmol l^{-1} is no longer followed by a re-polarization (n = 6).

Discussion

The effect of glibenclamide on fluid secretion rates

The existence of a functional link between the activity of the basolateral Na^+/K^+-ATPase and the basolateral K^+ conductance via K_{ATP} channels is well established in vertebrate epithelial cells (Grasset et al., 1983; Matsumura et al., 1984; Smith and Frizzell, 1984; Tsuchiya et al., 1992). At concentrations of 0.1 and 0.5 mmol l^{-1}, glibenclamide, a specific K_{ATP} channel blocker, decreases fluid secretion rates of stimulated *Tenebrio* tubules by 34 and 42% respectively (Fig. 1). The drug concentrations used are relatively high compared to those used to inhibit K_{ATP} channels of pancreatic β-islet cells, but are comparable to doses used in the renal proximal tubule (Tsuchiya et al., 1992). The reason for the apparent differences in sensitivity of K_{ATP} channels to glibenclamide is not clear, but appears to depend on the association of different sulfonylurea receptors with the K_{ATP} -channel unit (Benz and Kohlhardt, 1994). This is in accordance with the existence of a large family of K_{ATP} channels, which have, among other properties, different sensitivities to sulfonylureas (Ashcroft and Ashcroft, 1990).

The effect of glibenclamide on the basolateral membrane potential

The involvement of K_{ATP} channels in control Ringer (50 mmol l^{-1} K^+) seems to be indicated by the inhibition of fluid secretion by glibenclamide, although the substance had no visible effect on V_{bl} in control Ringer. The lack of response might be due to the masking of K_{ATP} channel activity by other highly conductive K^+ channels present in the basolateral membrane of insect tubules.

In low bath $[K^+]$ (5 mmol l^{-1}) glibenclamide had a detectable effect on V_{bl} similar to that previously observed with the K^+ channel blocker, Ba^{2+} (Wiehart et al., 2003). Depending on the putative electrochemical gradient for K^+, glibenclamide either caused a small but significant hyperpolarization of 3.6 ± 1.2 mV (Fig. 2A) or a significant depolarization of 9 ± 1.5 mV (Fig. 2B) of V_{bl}, indicating the inhibition of either inward (hyperpolarization) or outward (depolarization) K^+ movement through glibenclamide sensitive K^+ channels. The open probability of the K_{ATP} channels at bath

concentrations of 135 mmol l^{-1} NaCl and 5 mmol l^{-1} KCl must therefore be relatively high. This is supported by a patch-clamp study on rat cortical collecting tubules in which the authors found a bath concentration of 5 mmol l^{-1} KCl and 135 mmol l^{-1} NaCl to be optimal for K_{ATP} channels to be in an open state (Wang et al., 1990).

The addition of 6 mmol l^{-1} Ba^{2+} complements the initial response observed with glibenclamide by a further hyperpolarization or depolarization of V_{bl}, demonstrating the inward and outward electrochemical gradient for K^+ respectively (Fig. 2A, B). The sensitivity of this large family of K_{ATP} channels to Ba^{2+} is not clear. Tsuchiya et al. (1992) determined that K_{ATP} channels are almost exclusively responsible for the K^+ conductance in the renal proximal tubule. Blocking the conductive K^+ channels with glibenclamide caused a 95% inhibition in the basolateral membrane K^+ conductance compared to 84% when blocking with Ba^{2+}. This difference indicated that the K_{ATP} channels were less sensitive to Ba^{2+}. In line with this study, the K_{ATP} channels present in *Tenebrio* Malpighian tubule cells appear less sensitive to Ba^{2+}. The additional hyperpolarization from -51.8 ± 5.6 mV to -54.7 ± 5.8 mV ($n = 5$) or depolarization from -45 mV to -41 mV ($n = 1$) caused by glibenclamide in the presence of Ba^{2+} substantiates this.

However, caution must be exercised when interpreting results with glibenclamide, since this sulphonylurea compound has been shown to inhibit the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel (Sheppard and Welsh, 1992; Schultz et al., 1996), which is present in most secreting epithelia of vertebrates. Although this CFTR channel has not been characterized in insects, we cannot rule out its existence.

The effect of ouabain and glibenclamide on the basolateral membrane potential

Fluid secretion is inhibited by 1 mmol l^{-1} ouabain (Wiehart et al., 2003). However ouabain has no detectable effect on V_{bl} in control conditions (50 mmol l^{-1}). Possibly the presence of high conductance K^+ channels masked an effect on any other electrogenic process in the basolateral membrane. The effect expected when the

outward electrogenic pump current is blocked is a depolarisation of the membrane (Messner et al., 1985; Horisberger and Giebisch, 1988). This has been observed in unstimulated salivary glands of *Calliphora* (Berridge and Schlue, 1978) as well as in Malpighian tubule cells of *Drosophila* (Linton and O'Donnell, 1999). In low K^+, in the presence of Ba^{2+}, V_{bl} was affected: in 14 out of 16 cells, the membrane hyperpolarized in the presence of ouabain. Involvement of K_{ATP} channels was confirmed by applying ouabain after pretreatment with glibenclamide (in the presence of Ba^{2+}): the effect was reversed and in all experiments (n=8) ouabain depolarised V_{bl} (Fig 4A, B).

Glibenclamide changes the sensitivity of V_{bl} to the external $[K^+]$

In contrast to findings for the forest ant *Formica polyctena* (Weltens et al., 1992), the basolateral membrane of *Tenebrio* tubule cells does not appear to lose its sensitivity to the bath $[K^+]$ in the presence of 6 mmol l^{-1} Ba^{2+}. Increasing the bath $[K^+]$ from 5 mmol l^{-1} to 140 mmol l^{-1} K^+ resulted in an immediate depolarization of V_{bl}, with a mean value of 75.3 ± 2.4 mV (n = 6). However, this sudden depolarization was followed by a re-polarization of between 30 and 40 mV after 3 to 8 minutes (Fig. 5A). Again the involvement of the Na^+/K^+-ATPase and the K_{ATP} channels seems to be the explanation.

A rise in Na^+ transport and intracellular $[Na^+]$ is the primary physiological stimulus for the Na^+/K^+-ATPase in vertebrate tissue (Mauerer et al., 1998; Tsuchiya et al., 1992). In our study, a low bath $[K^+]$ (5 mmol l^{-1}) and therefore a high $[Na^+]$ (141 mmol l^{-1}) could be responsible for activating the Na^+/K^+-ATPase, thereby increasing the open probability of the K_{ATP} channels. The initial large depolarization seen when changing the bath $[K^+]$ from a low to a high value is most probably due to the following: (1) Ba^{2+}, being a competitive inhibitor of K^+ channels, is "knocked-off" by the inward flux of K^+ ions at high bath K^+ (Eaton and Brodwick, 1980; Armstrong and Taylor, 1980) and (2) the initial intracellular [ATP] is relatively low, which means the open probability of the K_{ATP} channels is high allowing an initial influx of K^+ ions. However, at a bath concentration containing no NaCl (140 mmol l^{-1} KCl), the Na^+/K^+ pump stops functioning, resulting in a time-dependent increase of $[ATP]_i$ and therefore the closing

of K_{ATP} channels. This might explain the observed re-polarization of V_{bl} after a few minutes: the apical V-ATPase increases the cell negative potential and compensation by K^+ entrance across the basolateral membrane is slowed down. Again this response was not seen in experiments without Ba^{2+}, indicating that other highly conductive K^+ channels mask the presence of K_{ATP} channels.

To substantiate the above hypothesis, experiments were repeated in the presence of Ba^{2+} and 0.5 mmol l^{-1} glibenclamide. The hyperpolarization of V_{bl} to a mean of –66 ± 6.2 mV, when $[K^+]$ was decreased, was far less than the -88.5 ± 3.4 mV when only Ba^{2+} was present. Moreover, although a substantial depolarization of V_{bl} was still seen when the bath $[K^+]$ was changed from 5 to 140 mmol l^{-1} (43 mV), this was considerably less than in experiments that involved Ba^{2+} alone. Most remarkable, however, was the sluggish response of V_{bl} in the presence of both substances. Both the hyperpolarization and depolarization of V_{bl} in response to a different bath $[K^+]$ were much slower, with the depolarization in some experiments taking more than 6 times longer than in earlier experiments with only Ba^{2+}. Moreover the depolarization of V_{bl} was no longer followed by a re-polarization, indicating that the putative K_{ATP} channels were blocked and therefore insensitive to the increase in $[ATP]_i$ expected when the Na^+/K^+-ATPase is inhibited by a decrease in Na^+. The final depolarization (after re-polarization), when only Ba^{2+} was present was 37 ± 4.9 mV (n = 6), and is comparable to the depolarization of 43 ± 5.7 mV (n = 6) when both substances were present, possibly indicating that the K_{ATP} channels are blocked in both instances. Another marked effect of glibenclamide was that the basolateral membrane became increasingly less responsive to the surrounding $[K^+]$ with time. Reintroduction of a low bath $[K^+]$ (5 mmol l^{-1}) still elicited a hyperpolarization of V_{bl}, but to a lesser extent. In experiments where only Ba^{2+} was present, V_{bl} stayed responsive to the bath $[K^+]$ (Fig. 5A).

In summary, the effects of glibenclamide, a K_{ATP} channel blocker, on both the fluid secretion rate and basolateral membrane potentials of *Tenebrio* Malpighian tubules are

strong indications of the presence of K_{ATP} channels and the involvement of these channels in ion transport.

Financial support was provided by a bilateral award (Bil98/53) under the Flemish-South African agreement on scientific and technological cooperation, and by the South African National Research Foundation and the University of Pretoria.

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Paper 6

The electrophysiological effects of the endogenous diuretic and antidiuretic peptides in the Malpighian tubules of *Tenebrio molitor*

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Journal of Insect Physiology (accepted, pending revision)

Note: **Paper 6** is written in the first person for thesis purposes.

Abstract

The effect of *Tenebrio molitor* diuretic (Tenmo-DH_{37}) and antidiuretic (Tenmo-ADFa) peptides and their respective second messengers (cyclic AMP and cyclic GMP) on basolateral (V_{bl}) and transepithelial (V_{te}) potentials of *Tenebrio* Malpighian tubules was determined using conventional microelectrodes. In the presence of 6 mmol l^{-1} Ba^{2+}, Tenmo-DH_{37} (100 nmol l^{-1}) reversibly hyperpolarized V_{bl} and depolarized V_{te}. A similar response was seen with the addition of 1 $mmol^{-1}$ cyclic AMP, however the apical membrane potential (V_{ap}) then showed a hyperpolarization, whereas a depolarization of V_{ap} was observed with Tenmo-DH_{37}. Bafilomycin A_1 (5 µmol l^{-1}) inhibited fluid secretion of stimulated tubules and reversed the hyperpolarization of V_{bl} in response to Tenmo-DH_{37}. In response to 100 nmol l^{-1} Tenmo-ADFa or 1 mmol l^{-1} cyclic GMP, V_{bl} and V_{te} depolarized, although cyclic GMP affected membrane potentials somewhat differently by causing an initial hyperpolarization of V_{bl} and V_{te}. In high [K^+]/-low [Na^+] Ringer, 1 mmol l^{-1} amiloride decreased fluid secretion rates, and depolarized both V_{bl} and V_{te}. Amiloride significantly decreased luminal pH in paired experiments, indicating the presence of a K^+/nH^+ exchanger in tubule cells of *Tenebrio*. The results suggest that the endogenous factors and their second messengers stimulate/inhibit fluid secretion by acting on the apical V-ATPase and basolateral K^+ transport.

Keywords: CRF-related peptides, diuretic peptide, antidiuretic peptide, Malpighian tubules, fluid secretion, *Tenebrio molitor*.

Introduction

Various factors that stimulate Malpighian tubule secretion have been isolated and characterized in a number of insect species. The largest group, the CRF-related peptides show some structural relationship to the vertebrate CRF/urotensin/sauvagine family of peptides and stimulate fluid secretion *via* an increase in intracellular cyclic AMP (Audsley et al., 1995). Since the first CRF-related diuretic peptide was isolated from the hawkmoth *Manduca sexta* (Kataoka et al., 1989), twelve other peptides belonging to this family have been isolated from different insect orders (for complete list see Baldwin et al., 2001), two of them from the beetle *Tenebrio molitor* (Furuya et al., 1995, 1998). Both Tenmo-DH_{37} and Tenmo-DH_{47}, containing 37 and 47 amino acid residues respectively, increase intracellular cyclic AMP and stimulate fluid

secretion at nanomole concentrations, Tenmo-DH_{37} being the more potent of the two (Wiehart et al., 2002).

The mechanism that drives fluid transport in insect Malpighian tubules has been widely accepted as a H^+-pumping V-ATPase located in the apical membrane. By creating a proton gradient and/or an electrical potential gradient across the apical membrane, this pump facilitates the movement of cations from the cell to the lumen *via* apical Na^+/nH^+ and/or K^+/nH^+ exchangers (Wieczorek et al., 1989; Wieczorek et al., 1991). Basolateral entry of cations occurs *via* a series of channels, possibly via the Na^+/K^+-ATPase or through the electroneutral, bumetanide-sensitive $Na^+/K^+/2Cl$ or furosemide-sensitive K^+/Cl^- co-transporters. Cyclic AMP has been shown to regulate the mechanism of fluid transport at different points. In *Rhodnius* and *Drosophila* tubules an increase in intracellular cyclic AMP increases cation transport *via* the apical V-ATPase (O'Donnell and Maddrell, 1984; O'Donnell et al., 1996) and in tubules of *Aedes* this second messenger increases transepithelial active transport of Na^+ by increasing the basolateral membrane Na^+ conductance (Williams and Beyenbach, 1984; Sawyer and Beyenbach, 1985; Beyenbach and Petzel, 1987; Petzel et al., 1987). In addition, Hegarty et al. (1991) reported an increase of transport *via* an electroneutral, bumetanide-sensitive cotransporter in cyclic AMP stimulated tubules of *Aedes*.

Endogenous antidiuretic factors that act directly on insect Malpighian tubules are less well known, but have been demonstrated in haemolymph and corpora cardiaca extracts of *Acheta* (Spring and Clark, 1990), whole body extracts of *Aedes* (Petzel and Conlon, 1991) and head extracts of *Formica* (De Decker et al., 1994; Laenen et al., 2001) and *Leptinotarsa* (Lavigine et al., 2001). These factors have however not been fully sequenced and the second messengers involved are unidentified. So far this has only been accomplished for the two antidiuretic peptides isolated from *Tenebrio* (Eigenheer et al., 2002; Eigenheer et al., in press). Tenmo-ADFa and ADFb consist of 14 and 13 amino acid residues respectively. Although these peptides are structurally unrelated, both inhibit fluid secretion in *Tenebrio* tubules through the second messenger cyclic GMP.

Previously I have shown the antagonistic effects of the endogenous diuretic and antidiuretic peptides and their second messengers by means of fluid secretion assays (Wiehart et al., 2002) and demonstrated the extensive localization of Tenmo-DH_{37} in the CNS and corpora cardiaca, supporting its function as a neurohormone (Wiehart et al., 2002). In addition, I identified the transport mechanisms involved in K^+ uptake during fluid secretion by *Tenebrio* tubules (Wiehart et al., 2003a,b).

I now extend this study by investigating the effect of these endogenous peptides on ion transport mechanisms. Our electrophysiological results suggest that fluid secretion/inhibition by these peptides is achieved by influencing at least three parameters simultaneously, the rate of H^+ extrusion by the V-ATPase, basolateral K^+ conductance, and possibly apical Cl^- conductance. Furthermore I investigate the presence of an amiloride-sensitive cation/nH^+ exchanger and its importance in fluid secretion and maintenance of cell pH.

Materials and Methods

Animals

Tenebrio larvae were kept under crowded conditions at room temperature (20-23°C) and fed on a diet of bran and apple. Care was taken in selecting mealworms of similar size for all experiments.

Artificial salines

The composition of the control Ringer (in mmol l^{-1}) was as follows (Nicolson, 1992): NaCl 90, KCl 50, $MgCl_2$ 5, $CaCl_2$ 2, $NaHCO_3$ 6, NaH_2PO_4 4, glycine 10, proline 10, serine 10, histidine 10, glutamine 10, and glucose 50. The pH was adjusted to 7.0 with HCl and the osmolality was kept at 390 mOsm kg^{-1}. Phenol red was incorporated into the Ringer as indicator. K^+-free solutions were obtained by replacing KCl with NaCl, and low-Na solutions by replacing NaCl with KCl and omitting the NaH_2PO_4 (low-Na solutions contained 6 mmol l^{-1} Na^+). Solutions were freshly prepared each week, filtered through 0.22 μm Millipore filters and kept at 2°C until used. The pH was measured daily before use. In experiments containing Ba^{2+}, NaH_2PO_4 was omitted from all salines to maintain constant osmolality and prevent precipitation of barium dihydrogen phosphate.

The following substances were tested at the concentrations given: barium, 6 mmol l^{-1} ($BaCl_2$; Janssen Chemica); bafilomycin A_1, 5 μmol l^{-1} (Alexis); amiloride, 1 mmol l^{-1} and 0.1 mmol l^{-1}; cyclic AMP, 1 mmol l^{-1}; cyclic GMP, 1 mmol l^{-1} (Sigma). The endogenous diuretic peptide, Tenmo-DH_{37} (tested at 100 nmol l^{-1}) and antidiuretic peptide Tenmo-ADFa (100 nmol l^{-1}) were a generous gift from Prof. D.A. Schooley, University of Nevada, Reno.

Fluid secretion experiments

The technique of measuring fluid secretion has been described previously (Wiehart et al., 2002). Briefly, free portions of the tubules were isolated and transferred into droplets (50 μl) of physiological saline, covered with water-saturated liquid paraffin in a Sylgard-lined Petri dish. The ends of each tubule were pulled out of the bathing fluid and wrapped around Minuten pins, where they continued to secrete. Droplets of secreted fluid were removed with a fine glass pipette and their diameters measured with a calibrated eyepiece graticule. The volume, and therefore the rate of secretion, was determined assuming the droplets to be spherical. The tubules were allowed to equilibrate for 20 min before three control readings were made at 15 min intervals. The bathing solution was then replaced with the experimental solution containing the test substances. Measurements were taken over a 45-60 min period. Rates of secretion were expressed as a percentage of the third control rate reading. Each experiment included five to eight replicates.

Measurement of basolateral (V_{bl}) and transepithelial (V_{te}) potential differences

This method has been described in detail previously (Wiehart et al., 2003a). In short, a portion of a Malpighian tubule (3-5 mm) was suspended in a Ringer bath. To facilitate impalements, the tubule was held in place by two holding pipettes. Intracellular (V_{bl}) and transepithelial (V_{te}) measurements were performed with 3M KCl-filled microelectrodes (Borosilicate filament glass, Harvard; OD 1.2 mm, ID 0.69 mm; tip diameter < 0.5 μm; resistance 20-40 MΩ), connected to a Micro Probe System M-707 electrometer via a Ag/AgCl wire. Cell impalement was accepted if a sudden drop in potential occurred; if the potential was stable for at least a few minutes; and if the electrode potential differed by not more than 3 mV from the baseline after

withdrawal. V_{te} was measured by advancing the microelectrode through the cell layer into the lumen of the tubule. The apical membrane potential (V_{ap}) was calculated as the difference between the measured V_{te} and V_{bl}.

pH measurements of secreted fluid

pH was measured with a coated wire pH electrode (Beetrode; World Precision Instruments). Calibration solutions for pH measurements were made by adjusting control Ringer (50 mmol l^{-1} K^+; 90 mmol l^{-1} Na^+) to pH 6.5, 7.0 and 7.5 using either HCl or NaOH . In these experiments the electrode was calibrated before and after pH measurements on the secreted fluid by placing the saline solutions under the liquid paraffin next to the bathing drop in the fluid secretion assay. The slope of the electrode was 54 mV per decade ($r^2 = 0.984$).

Statistics

Results are presented as mean values ± S.E.M. with the number of tubules (t) or number of measurements (n) in parentheses. The statistical significance of differences in fluid secretion or electrical potentials was evaluated by paired or unpaired Student's t-tests (two-tailed). A value of $P < 0.05$ was accepted as statistically significant.

Results

The effect of DH_{37} and cyclic AMP on V_{bl} and V_{te}

In control Ringer (50 mmol l^{-1} K^+), neither DH_{37} (100 nmol l^{-1}) nor cyclic AMP (1 mmol l^{-1}) had any visible effect on V_{bl} or V_{te} (n = 2-4). In the presence of Ba^{2+}, 100 nmol l^{-1} DH_{37} hyperpolarized V_{bl} from -61.9 ± 4.1 mV to -72.8 ± 3.8 mV (n = 13; $P < 0.0001$) and V_{te} depolarized from 24.5 ± 6.3 to 6.4 ± 1.1 mV (n = 8, $P = 0.02$). Fig. 1A shows the result of a typical experiment.

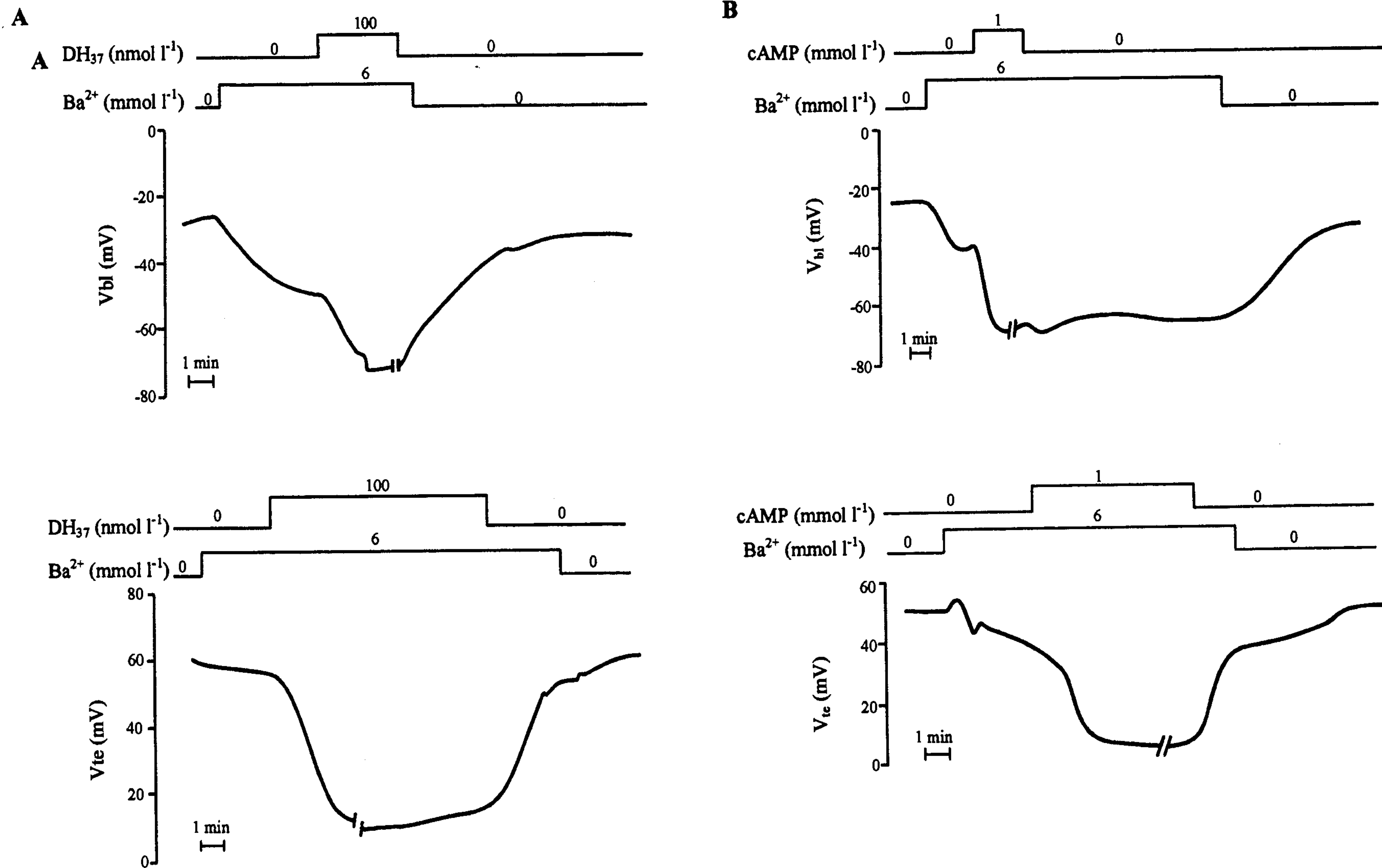

Fig. 1. The effect of DH_{37} (**A**) and cyclic AMP (**B**) on V_{bl} and V_{te} of *Tenebrio* tubules. In the presence of 6 mmol l^{-1} Ba^{2+}, addition of 100 nmol l^{-1} DH_{37} reversibly hyperpolarized V_{bl} by an average of 11 mV (n = 13; $P < 0.0001$). V_{te} dropped significantly by an average of 18 mV (n = 8; $P < 0.02$), but recovered slowly after washout of DH_{37}. Cyclic AMP mimicked this response by hyperpolarizing V_{bl} by an average of 15 mV (n = 15; $P < 0.001$) and dropping V_{te} by 10 mV (n = 14; $P < 0.001$).

Cyclic AMP (1 mmol l^{-1}) mimicked the effect of DH_{37} by hyperpolarizing V_{bl} from -49.8 ± 3.1 mV to -64.5 ± 3.6 mV (n = 15; $P < 0.001$) and causing a drop in V_{te} from 18.8 ± 2.4 mV to 8.0 ± 0.5 mV (n = 14; $P < 0.001$; Fig. 1B). The effects of DH_{37} and cyclic AMP on V_{bl}, V_{te} and V_{ap} are summarized in Fig. 2A,B. After washout of either stimulant the potentials dropped back to control values.

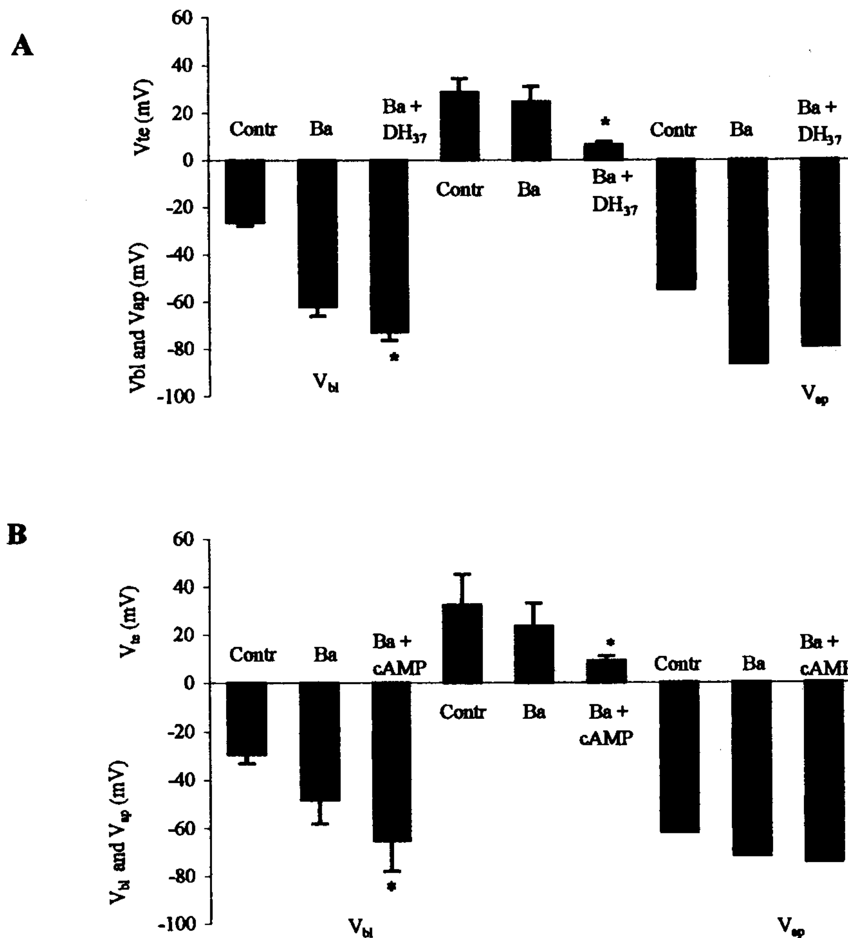

Fig. 2. Summary of the effect of DH_{37} (A) and cyclic AMP (B) on the electrical potentials of *Tenebrio* tubules. In the presence of Ba^{2+}, V_{bl} hyperpolarized and V_{te} depolarized significantly in response to either 100 nmol l^{-1} DH_{37} or 1 mmol l^{-1} cyclic AMP. However, V_{ap} depolarized in response to the endogenous factor, but hyperpolarized in the presence of cyclic AMP (n = 13 - 15; * significantly different from control with barium treatment, $P < 0.05$).

The effect of Tenmo-ADFa and cyclic GMP on V_{bl} and V_{te}

The endogenous antidiuretic peptide Tenmo-ADFa and its second messenger cyclic GMP inhibit fluid secretion rates of *Tenebrio* tubules (Wiehart et al., 2002). The effect on V_{bl} and V_{te} of both inhibitors was investigated in the presence of 6 mmol l^{-1}

Ba^{2+}. Tenmo-ADFa (100 nmol l^{-1}) caused a slow depolarisation of V_{bl} from -47.5 ± 3.7 to -43.6 ± 4 mV (Fig. 3A; n = 14; $P < 0.007$) and an increase in V_{te} from 24.3 ± 5.4 to 30.3 ± 6.8 mV (Fig. 3A; n = 6; $P = 0.03$). The potentials returned to control values after washout of both inhibitor and Ba^{2+}. Cyclic GMP (1 mmol l^{-1}) caused an initial hyperpolarization of V_{bl} from -46.6 ± 3.9 to –57.0 ± 5.1 mV within 3 min, after which the potential depolarised again to –36.0 ± 4.5 mV (Fig. 3B; n = 5; $P < 0.005$). V_{te} followed this response with an initial drop in potential from 36 ± 4.9 to 31.3 ± 3.5 mV within 3 min, after which the potential increased again to 59.3 ± 5.4 mV (Fig. 3B; n = 3; $P < 0.01$). Fig. 4 A, B summarizes the final results obtained for cyclic Tenmo-ADFa and GMP.

The effect of bafilomycin A_1 on fluid secretion of stimulated Tenebrio tubules

The effect of bafilomycin A_1, a blocker of the V-ATPase, was investigated on Malpighian tubules previously stimulated by 1 mmol l^{-1} cyclic AMP. The mean fluid secretion rate after 45 min of cyclic AMP stimulation was 12.3 ± 1.4 nl/min, in 8 tubules, each serving as its own control. After addition of 5 µmol l^{-1} bafilomycin A_1 to the peritubular bath, fluid secretion rates declined to 1.9 ± 0.24 nl min^{-1} in 45 min. Secretion rates recovered partially when bafilomycin was removed from the bath droplet, reaching 7.4 ± 0.93 nl/min after 60 min (Fig. 5).

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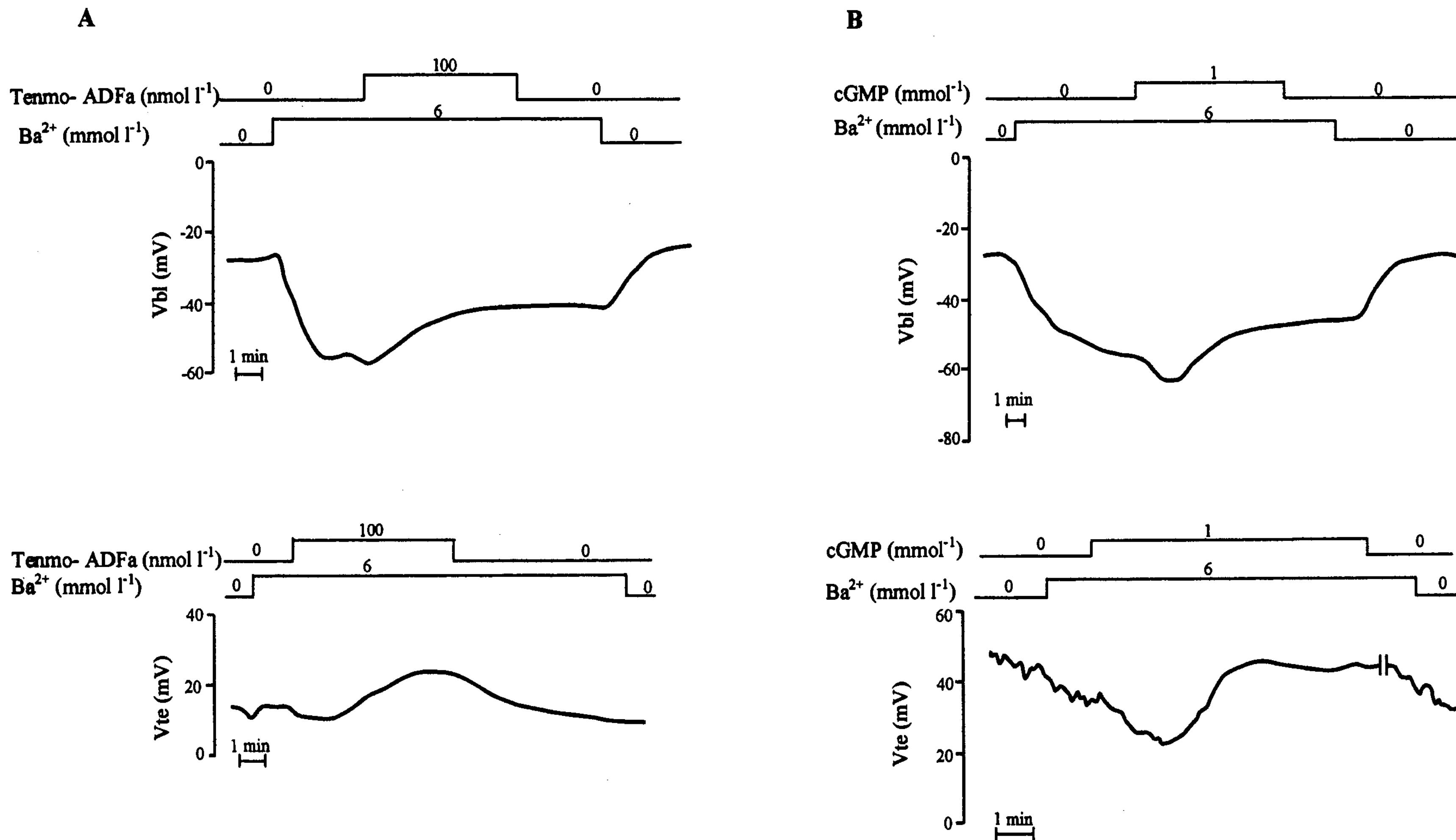

Fig. 3. Typical experiments showing the effect of 100 nmol l^{-1} Tenmo-ADFa (**A**) and 1 mmol l^{-1} cyclic GMP (**B**). Both V_{bl} and V_{te} depolarized in the presence of Tenmo-ADFa (n = 14 and 6 respectively). Cyclic GMP causes an initial hyperpolarization of V_{bl} and V_{te} within 3 min, which is followed by a depolarization of both potentials (n = 5 and 3 respectively).

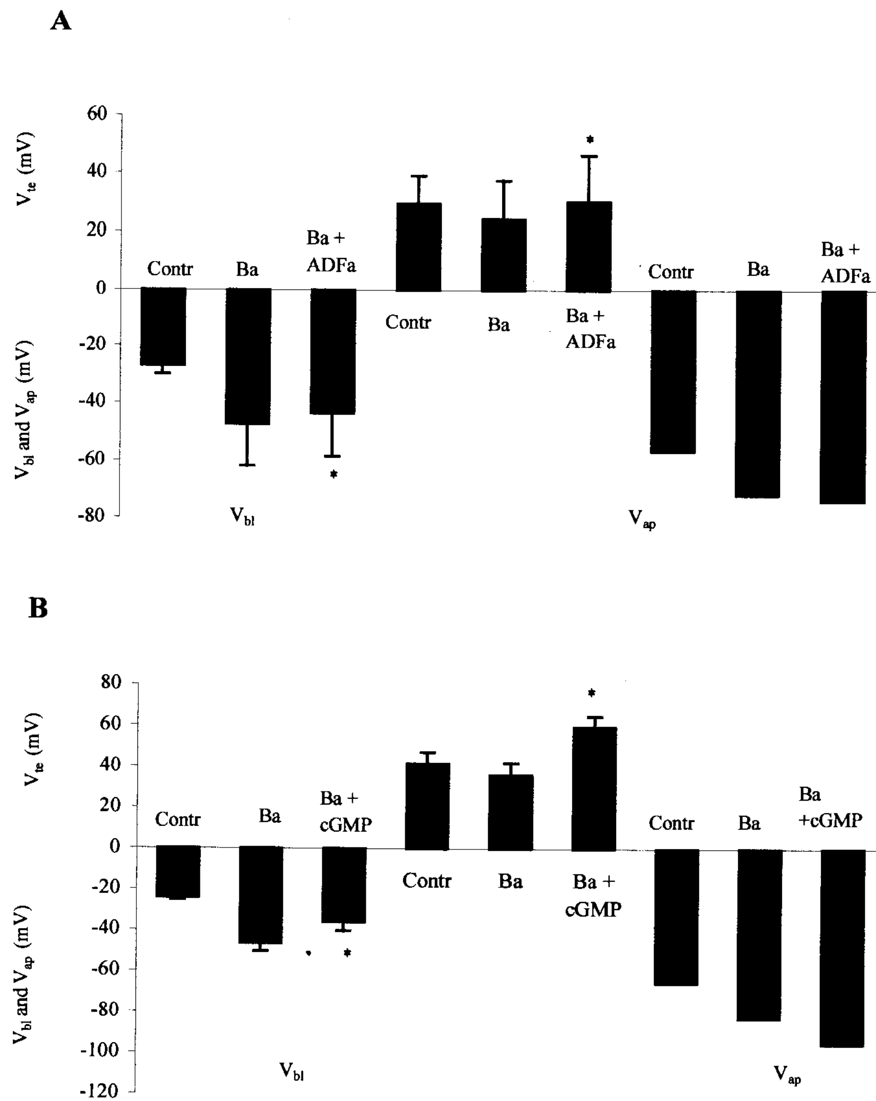

Fig. 4. Summary of the effect of Tenmo-ADFa (A) and cyclic GMP (B) and on the electrical potentials of *Tenebrio* tubules. Both Tenmo-ADFa and cyclic GMP caused a depolarization of V_{bl} and a hyperpolarization of with no significant change in V_{ap} (n = 14 and 6, respectively and 5 and 3, respectively; * indicates significantly different, $P < 0.05$).

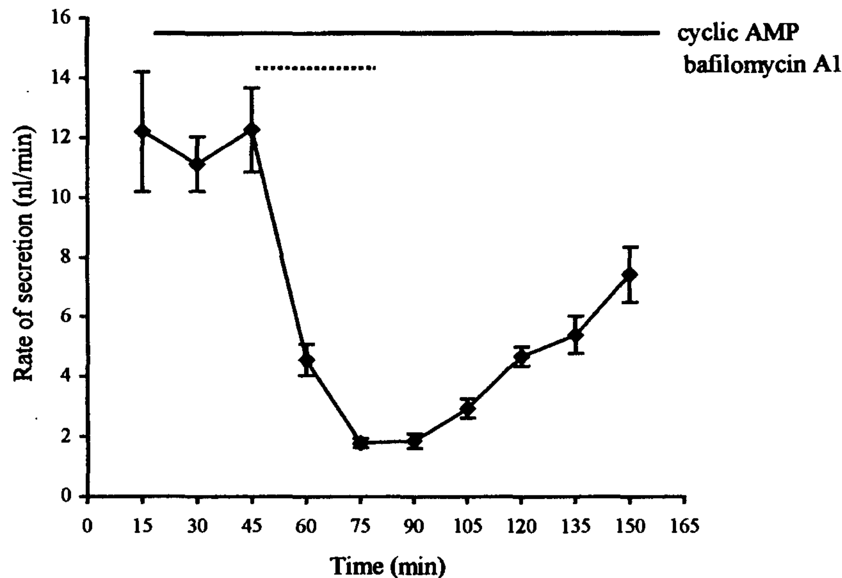

Fig. 5. The effect of 5 μmol l^{-1} bafilomycin A_1 on the fluid secretion rate of stimulated Malpighian tubules. Addition of bafilomycin A_1 to cyclic AMP stimulated tubules decreased the fluid secretion rate by approximately 85% (n = 8 tubules). The horizontal bars indicate the time of exposure to cyclic AMP and bafilomycin A_1.

The effect of bafilomycin A_1 on V_{bl} and V_{te}

Addition of 5 μmol l^{-1} bafilomycin A_1 in the presence of 6 mmol l^{-1} Ba^{2+} caused a slow and irreversible depolarisation of both V_{bl} and V_{te}; V_{bl} depolarized by 7.3 ± 2.3 mV after 10 min and V_{te} dropped by 14.5 ± 3.2 mV over the same time period (n = 4 in both instances; result not shown). In addition I investigated the effect of bafilomycin A_1 on V_{bl} in the presence of the endogenous diuretic peptide, DH_{37}. The hyperpolarization of V_{bl} by 100 nmol l^{-1} DH_{37} seen in the presence of 6 mmol l^{-1} Ba^{2+} was reversed by the addition of 5 μmol l^{-1} bafilomycin A_1 (Fig. 6; n = 5).

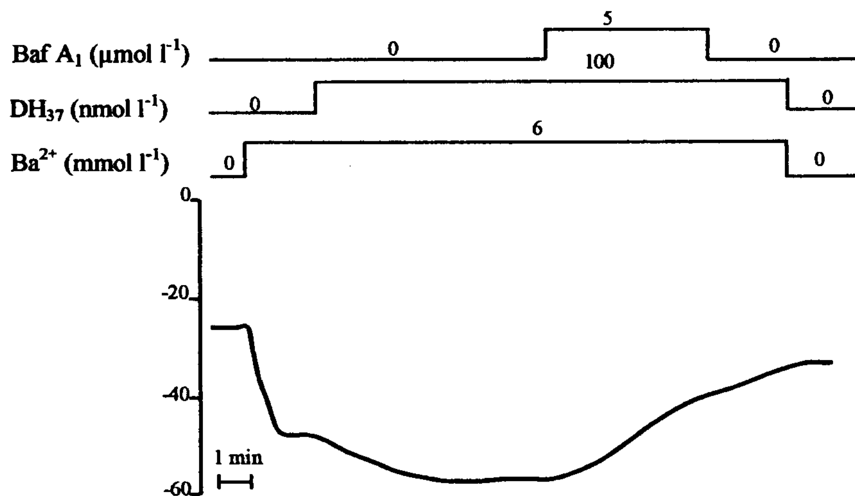

Fig. 6. Response of V_{bl} to bafilomycin A_1 in the presence of DH_{37} and Ba^{2+}. The hyperpolarization of V_{bl} by DH_{37} in the presence of Ba^{2+} is reversed with the addition of 5 µmol l-1 bafilomycin to the peritubular bath (n=5).

The effect of amiloride on tubule secretion rates

At millimolar concentrations, amiloride has a broad effect and is known to influence the Na^+/H^+ exchanger present in the apical membrane (Hegarty et al., 1992). However, at these concentrations amiloride also blocks Na^+ channels. Fluid secretion experiments were therefore carried out in the presence of a high bath $[K^+]$ (140 mmol l^{-1}; 6 mmol l^{-1} Na^+). Previously I have shown that a high $[K^+]$ stimulates fluid secretion in *Tenebrio* tubules (Wiehart et al., 2003a). Addition of 1 mmol l^{-1} amiloride significantly decreased fluid secretion rates from 3.89 ± 0.66 to 0.98 ± 0.11 nl min^{-1} (n = 5; Fig. 7). The secreted fluid became notably more acidic, visible as a result of the phenol red incorporated into the Ringer solution. Fluid secretion rates recovered slowly to 1.87 ± 0.26 nl min^{-1} within 60 min of washout.

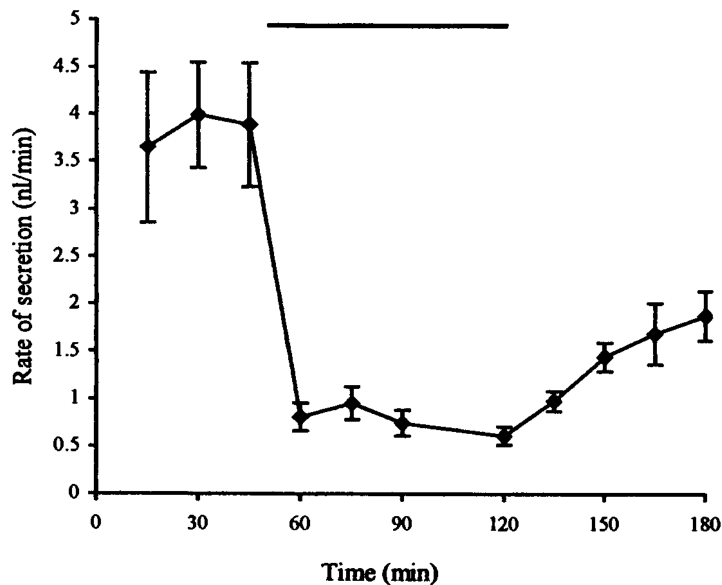

Fig. 7. The effect of 1 mmol l^{-1} amiloride on the fluid secretion rate of *Tenebrio* tubules. In high bath K^+ (140 mmol l^{-1} K^+; 6 mmol l^{-1} Na^+), amiloride reversibly decreased fluid secretion rates (mean ± S.E.M., n=5 tubules). The horizontal bar indicates the time of exposure to amiloride.

The effect of amiloride on V_{bl} and V_{te}, in the presence of Ba^{2+}

To examine how amiloride influences ion movement in *Tenebrio* tubules I investigated the effect of 0.1 mmol l^{-1} and 1 mmol l^{-1} amiloride on V_{bl} and V_{te} in control Ringer (50 mmol l^{-1} K^+) and 1 mmol l^{-1} amiloride in high bath K^+ (140 mmol l^{-1} K^+, 6 mmol l^{-1} Na^+). Ba^{2+} (6 mmol l^{-1}) was included in all experiments. Table 1 summarizes the results. In all experiments the addition of amiloride caused a depolarization of V_{bl} and V_{te}. Figure 8 shows a typical experiment depicting the response of V_{bl} and V_{te} to 1 mmol l^{-1} amiloride in 140 mmol l^{-1} K^+. The effect of amiloride was completely reversible after washout (not shown).

$[K^+]_{bath}$ (mmol l^{-1})	Amiloride (mmol l^{-1})	V_{bl} (mV)		V_{te} (mV)		V_{ap} (mV)		n
		Control	Amiloride	Control	Amiloride	Control	Amiloride	
50	0.1	-53 ± 3.1	-46.0 ± 2.6 *	16 ± 3.5	11 ± 1.0	-69.3 ± 3.3	-57 ± 1.3	3
50	1	-47.4 ± 2.9	-26.7 ± 3.3 *	18.2 ± 3.6	7.5 ± 1.2 *	-65 ± 3.3	-34.2 ± 2.3 *	7
140	1	-38.4 ± 6.2	-19.4 ± 2.7 *	27.8 ± 3.4	14.5 ± 4.8 *	-66.2 ± 4.8	-33.9 ± 3.8 *	5

Table 1. The effect of amiloride in the presence of Ba^{2+} on the electrical potentials of the Malpighian tubules of *Tenebrio*. (* Significantly different, $p < 0.05$)

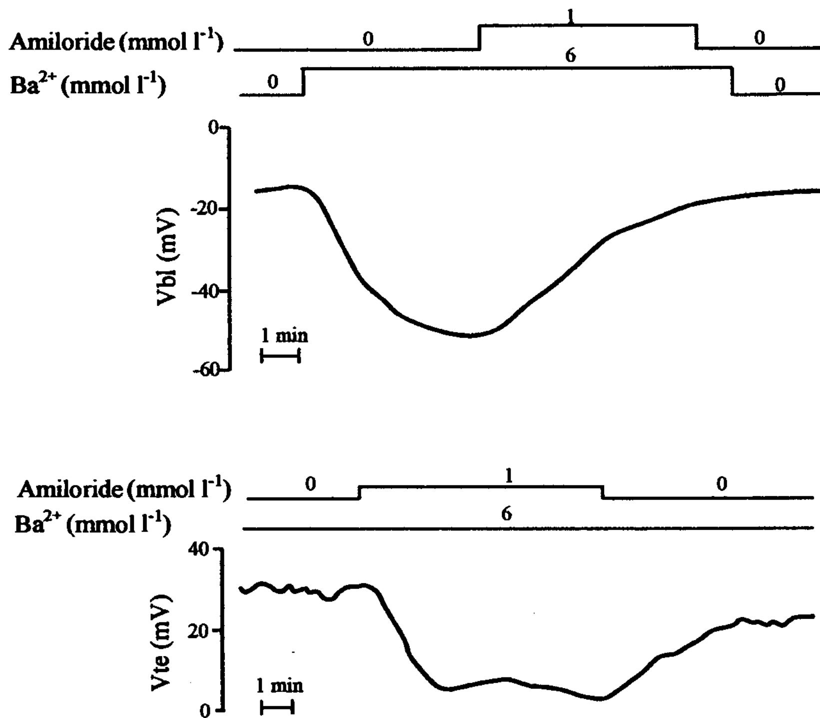

Fig. 8. Typical experiment showing the effect of amiloride on V_{bl} and V_{te} in high bath K^+ (140 mmol l^{-1} K^+; 6 mmol l^{-1} Na^+). In the presence of 6 mmol l^{-1} Ba^{2+}, 1 mmol l^{-1} amiloride depolarized V_{bl} significantly (n=5; P<0.001) and also caused a significant drop of V_{te} (n=5; P<0.01).

The effect of amiloride on luminal pH

The pH measurements on the secreted fluid under control conditions were made over a 20 min period. The luminal pH in control conditions was 7.32 ± 0.05 (n = 7). Upon applying 0.1 mmol l^{-1} amiloride to the bath, the secreted droplet was removed and a new drop was allowed to build up during the next 20 min. The pH decreased significantly to 7.08 ± 0.04 in paired experiments (n = 7; $P < 0.007$).

Discussion

Tenmo-DH37 and cyclic AMP act on the apical V-ATPase, K^+ and possibly Cl^- transport

Fluid secreted by Malpighian tubules of *Tenebrio* is rich in K^+ and an increase in peritubular [K^+] increases the secretion rate (Wiehart et al., 2003a). It therefore is reasonable to hypothezise that the endogenous diuretic hormone Tenmo-DH37 and its second messenger cyclic AMP increase fluid secretion by increasing transepithelial transport of K^+ and Cl^- in *Tenebrio* tubules.

Weltens et al. (1992) have shown that the apical electrogenic V-ATPase in ant Malpighian tubules increases the cell negative potential across the basal membrane, thereby creating a small cell inward electrochemical gradient for K^+. An increase in K^+ conductance on its own is expected to depolarise the basolateral membrane, whereas a stimulatory effect on the pump increases the epithelial circuit current and would result in a hyperpolarization. Our results for *Tenebrio* tubules showed no visible effect on V_{bl} and only a small drop in V_{te} when Tenmo-DH_{37} (100 nmol l^{-1}) or cyclic AMP (1 mmol l^{-1}) were added in the absence of Ba^{2+}. Blocking the K^+ conductance with Ba^{2+} is expected to unmask the pump current (Weltens et al., 1992). When Ba^{2+} was included, both stimulants hyperpolarized V_{bl} and caused a significant drop in V_{te}, similar in time and magnitude. An increase in the rate of H^+ extrusion by the apical V-ATPase may render the cell interior more negative, resulting in a hyperpolarization of V_{bl} in the presence of Ba^{2+}. At the same time, V_{ap} remains fairly constant in the presence of cyclic AMP or decreases significantly in response to Tenmo-DH_{37}, depolarising up to 12 mV in one paired experiment. Possibly this is due to an increased Cl^- conductance across this membrane. Cyclic AMP has been shown to regulate Cl^- channels (Greger and Kunzelman, 1990) and an increased net lumen-directed Cl^- flux would shift V_{te} to a less positive potential. Our results correspond with this prediction, however it is not clear what pathway Cl^- follows; *via* apical Cl^- channels or the paracellular shunt (Beyenbach, 1995). In *Glossina* Malpighian tubules, cyclic AMP reduced transepithelial resistance, an effect attributed to an opening of apical Cl^- channels (Isaacson and Nicolson, 1994), whereas in the ant, the depolarisation of V_{te} was ascribed to a rise in Cl^- conductance *via* a Cl^- selective shunt (Dijkstra et al. (1994). The increase in fluid secretion, i.e. the increase of K^+ and Cl^- movement across the apical membrane, must be sustained by an increase of K^+ and Cl^- uptake across the basolateral membrane, possibly *via* the $Na^+/K^+/2Cl$ cotransporter (Hegarty et al., 1991; Ianowski and O'Donnell, 2001). Bumetanide, a blocker of the $Na^+/K^+/2Cl$ cotransporter, inhibits fluid secretion rates of stimulated and unstimulated *Tenebrio* tubules, but due to the electroneutral nature of this transporter no effect on electric potentials was seen (Wiehart et al., 2003a). Another possible route of basolateral K^+ entry may be the previously proposed K_{ATP} channels (Wiehart et al.,

2003b). These K^+ channels are activated by endogenous cAMP-dependent protein kinase (Mauerer et al., 1998), but further investigation is required to substantiate this.

Effects of cyclic AMP on Malpighian tubules vary with insect species. In tubules of *Onymacris, Locusta* and *Formica* V_{bl} remained unchanged in the presence of cyclic AMP while V_{ap} decreased (Nicolson and Isaacson 1987; Fogg et al., 1989; De Decker et al., 1990). These studies were conducted in the absence of Ba^{2+} and it is possible that the lack of a visible response of V_{bl} is due to the high conductance of the basolateral membrane, as in *Tenebrio* tubules. In tubules of *Aedes*, cyclic AMP depolarised V_{bl} and increased V_{te} to a more positive potential (Sawyer and Beyenbach 1985). At the same time the fractional resistance of the basolateral membrane decreased (Petzel et al., 1987), indicating an increased basolateral Na^+ conductance. This is in contrast to our results and those of O'Donnell and Maddrell (1984), who showed by means of intracellular recordings in *Rhodnius* that the changes in transepithelial potentials in response to cyclic AMP are entirely accounted for by the changes across the apical surface.

The effect of endogenous diuretic peptides on ion transport is less well studied. Fogg et al. (1989) compared cyclic AMP and corpora cardiaca extracts and found a small hyperpolarization of V_{bl} in *Locusta* tubules in response to both, but V_{te} and V_{ap} increased in the presence of cyclic AMP, but decreased in response to corpora cardiaca extracts. It is to be expected that membrane potentials would respond differently to cyclic AMP compared to crude tissue extracts as these contain numerous factors. This has *also been reported for Malpighian tubules of Aedes, Onymacris and Culex* salinarius (Williams and Beyenbach 1984; Nicolson and Isaacson 1987; Clark et al., 1998).
Although cyclic AMP and Tenmo-DH_{37} exert similar effects on V_{bl} and V_{te}, V_{ap} showed a non-significant hyperpolarization in the presence of cyclic AMP, but a significant depolarisation in response to DH_{37} (Fig 2 A,B); with a marked depolarisation of 16 mV in one paired experiment. The reason for the different response of V_{ap} to cyclic AMP and Tenmo-DH_{37} is not clear and, like Fogg et al. (1989), I have to conclude that cyclic AMP alone cannot mediate the full effects of the endogenous diuretic hormone.

Effect of bafilomycin A_1 and DH_{37}: evidence for a V-ATPase

Wieczorek et al. (1989) first discovered the presence of a vacuolar-type H-ATPase in the midgut epithelium of *Manduca sexta*, after which Bertram et al. (1991) indirectly confirmed its presence in Malpighian tubules of *Drosophila* by inhibiting fluid secretion rates with the specific V-ATPase inhibitor, bafilomycin A_1. This antibiotic also stops fluid secretion in tubules of *Formica* (Weltens et al., 1992), *Onymacris* and *Glossina* (see Nicolson 1993) and *Aedes* (Pannabecker and Beyenbach, 1993). *Tenebrio* is no exception and cyclic AMP-stimulated secretion rates were strongly inhibited by bafilomycin A_1 (Fig 5). The inhibitory effect was partially reversible and secretion rates recovered slowly after bafilomycin A_1 was washed out.

Bafilomycin A_1 alone had no effect on V_{bl} in *Tenebrio.* However, in the presence of Ba^{2+} depolarisation of both membrane potentials was observed. Similar results were seen in tubules of *Formica* (Weltens et al., 1992). The hyperpolarization of V_{bl} in response to Ba^{2+} and Tenmo-DH_{37} was reversed by bafilomycin A_1. Based on these results I suggest (1) the presence of a V-ATPase in the Malpighian tubules of *Tenebrio* and (2) that Tenmo-DH_{37} acts on the apical V-ATPase, among other transport mechanisms.

The effect of Tenmo-ADFa and second messenger cGMP

Two potent antidiuretic peptides have been isolated and characterized from *Tenebrio*, Tenmo-ADFa (Eigenheer et al., 2002) and ADFb (Eigenheer et al., in press). Both increase the intracellular cyclic GMP concentration in Malpighian tubule cells and decrease fluid secretion rates of isolated *Tenebrio* tubules, with EC_{50} values in the femtomolar region. Recently Lavigne et al. (2001) and Laenen et al. (2001) have reported the isolation of partially purified antidiuretic factors from the Colorado potato beetle, *Leptinotarsa decemlineata* and the forest ant, *Formica polyctena,* respectively. However, in both these studies the secondary messenger involved was not characterized. Very little is known about the mode of action of these endogenous antidiuretic factors and ultimately how they control ion transport to bring about inhibition of fluid secretion in isolated Malpighian tubules. In tubules of *Tenebrio,* hardly any change in electrical potential was seen with the addition of either inhibitor in the absence of Ba^{2+}. In the presence of Ba^{2+}, Tenmo-ADFa elicited a somewhat different response in V_{bl} and V_{te} compared to that of cyclic GMP. The endogenous

antidiuretic factor depolarised V_{bl} and increased V_{te} with no significant change in V_{ap} (Fig 4A). In essence this is the opposite response to that seen with of the endogenous diuretic peptide and I therefore suggest that Tenmo-ADFa inhibits fluid secretion by slowing the apical V-ATPase activity. This decrease in apical net transport most probably coincides with a decreased basolateral K^+ and Cl^- uptake. In *Formica* tubule cells the endogenous antidiuretic factor FopADF depolarised both V_{bl} and V_{te} in the absence of Ba^{2+}, resulting in a significant depolarization of V_{ap} (Laenen et al., 2001). These authors suggested a model whereby FopADF inhibits the active K^+ secretion by blocking both the apical proton pump and basolateral membrane K^+ channels.

The depolarization of V_{bl} and V_{te} by the second messenger, cyclic GMP, was in line with the effects of Tenmo-ADFa, but the response was biphasic. In the presence of Ba^{2+}, cyclic GMP initially increased both V_{bl} and V_{te} within 3 min, followed by a decrease of both (Fig. 3B); V_{ap} hyperpolarized throughout this biphasic response (Fig. 4B). The reason for this is not clear. The difference in response may be due to the fact that cyclic GMP needs to first cross the cell membrane to initiate an effect, in contrast to Tenmo-ADFa, which activates a receptor on the cell membrane and the intracellular signalling events may therefore occur much faster and in an orchestrated fashion. The different response of V_{ap} as well as the initial hyperpolarization of both V_{bl} and V_{te} in response to cyclic GMP may be indications that the second messenger alone, like cyclic AMP, is not able to mediate the full effect of the antidiuretic peptide.

Effects of amiloride: evidence for a K^+/nH^+ exchanger

In every insect studied so far, amiloride inhibits tubule fluid secretion (*Drosophila*, Bertram, 1989; *Aedes*, Hegarty et al., 1992; *Rhodnius*, Maddrell and O'Donnell, 1992; *Glossina*, Gee, 1976; *Locusta*, Fathpour and Dahlman, 1994). In all but two of these studies the authors concluded that amiloride affects Na^+ channels, whereas in the two bloodsuckers, *Aedes* and *Rhodnius*, the K^+/nH^+ and/or Na^+/nH^+ exchanger was suggested as the site of inhibition. Due to its dose-dependent effects (at doses of < 1 $\mu mol\ l^{-1}$ the effect is on the Na^+ channel; from 100 $\mu mol\ l^{-1}$ the effects are also on cation/nH^+ exchange), results with amiloride must be interpreted with caution (Petzel, 2000).

In isolated *Tenebrio* tubules the addition of 1mmol l^{-1} amiloride to Ringer containing a high [K^+] and low [Na^+] (140 mmol l^{-1} K^+, 6 mmol l^{-1} Na^+) decreased fluid secretion rates significantly. This is in contrast to findings in the salivary gland of *Calliphora* where the production of saliva was not affected by amiloride in high K^+ Ringer (120 mmol l^{-1} K^+, 55 mmol l^{-1} Na^+), although in control Ringer (20 mmol l^{-1} K^+, 155 mmol l^{-1} Na^+) fluid production fell dramatically (Berridge et al., 1976). In the latter study, amiloride was evidently specific for Na^+ transport. In *Locusta* tubules where the prime mover of fluid secretion is K^+, Fathpour et al. (1983) suggested that the reduction of intracellular Na^+ by amiloride may have an impact on the normal function of the basolateral Na^+/K^+-ATPase and ultimately reduce K^+ transport across the apical membrane. Our results do not support this hypothesis, since the use of high [K^+] and low [Na^+] Ringer should impair the normal function of the Na^+/K^+-ATPase, yet fluid secretion is stimulated by these concentrations (Wiehart et al., 2003b). Instead I suggest that amiloride (0.1 mmol l^{-1} and 1 mmol l^{-1}) blocks the apical K^+/nH^+ exchanger rather than channels in *Tenebrio* tubules. Our electrophysiological and pH results support this hypothesis. In the absence of Ba^{2+} no significant change in V_{bl} and V_{te} was seen in the response to amiloride. This was also found in tubules of *Onymacris* (Nicolson and Isaacson 1987). However, in the presence of Ba^{2+}, addition of 1 mmol l^{-1} amiloride to a high [K^+] bath depolarised all potentials significantly (Fig. 8), indicating that amiloride targets a transport mechanism located in the apical membrane (Weltens et al., 1992).

Depolarisation of V_{bl} and V_{te} in response to amiloride was also observed in tubules of *Drosophila* (Wessing et al., 1993). As in the latter study, the reason for the depolarisation of V_{bl} is not clear, since the inhibition of the K^+/nH^+ exchanger should result, if anything, in a hyperpolarization of V_{bl}. For this reason I suggest that amiloride has an indirect effect on the normal action of the apical V-ATPase. In insect tubules, luminal acidification is countered by the K^+/nH^+ and/-or Na^+/nH^+ exchange mechanism, recycling the protons and keeping the cellular and luminal pH fairly constant. In *Tenebrio* tubules, amiloride caused a drop in luminal pH from 7.32 ± 0.05 to 7.08 ± 0.04. Acidification of the lumen was also seen in amiloride-exposed tubules of *Drosophila* (Wessing et al., 1993). The resultant steeper electrochemical gradient

against which the V-ATPase has to function may cause H^+ extrusion to occur at a slower rate, resulting in the depolarization of V_{bl} observed.

In summary, the present study proposes that the endogenous diuretic and antidiuretic peptides of *Tenebrio*, as well as their second messengers, influence tubule secretion by acting on (1) the apical bafilomycin-sensitive V-ATPase, (2) Cl^- transport mechanisms and (3) basolateral K^+ conductance. Furthermore, fluid secretion assays and electrophysiological data demonstrate the existence and the importance of the cation/H^+ exchange mechanism in both fluid secretion and pH regulation.

Financial support was provided by a bilateral award (Bil98/53) under the Flemish-South African agreement on scientific and technological cooperation, and by the South African National Research Foundation and the University of Pretoria.

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GENERAL SUMMARY AND CONCLUSION

The regulation of fluid secretion by insect Malpighian tubules is complex. Since synthetic hormones became available in the 1990s, studies in this field have broadened and the complexity of hormonal control of fluid secretion has become apparent. In general, it appears that there is more than one type of diuretic hormone controlling ion transport and diuresis in most insect species studied thus far. The interaction of these different hormones makes primary urine formation by insect Malpighian tubules an intricate, but precisely controlled process. In this study the localization of endogenous factors in the beetle *Tenebrio molitor*, the control of Malpighian tubule secretion by these factors and the cellular mechanisms were analysed.

I. *Antagonistic control of fluid secretion in Tenebrio tubules*

The regulation of fluid secretion by endogenous and exogenous factors that act directly on *Tenebrio* Malpighian tubules was investigated (see paper 1).

The two CRF-related peptides Tenmo-DH_{37} and DH_{47}, isolated from *Tenebrio* head extracts' increase the intracellular cyclic AMP concentration (Furuya et al., 1995, 1998). In the current study it was demonstrated that both these factors stimulate fluid secretion of *in vitro* tubule preparations, but the response is neither synergistic nor additive. The reason for this is not clear, nor is it clear why a xeric insect like the mealworm would have two potent diuretic peptides. In the desert beetle, *Onymacris*, there is evidence that diuretic factors increase recycling of fluid to the haemolymph and Nicolson (1991, 1992) suggested that these peptides should rather be termed clearance hormones, as they redirect the tubule fluid anteriorly to moisten the midgut contents and filter the haemolymph of toxic waste, without loss of water. This was evident in experiments in which amaranth dye was injected into the haemolymph of mealworm larvae; dye could be seen in the midgut after a few minutes (Wiehart, personal observations).

Tenebrio can survive on dry bran, taking up the moisture present in the food and maintaining water balance by minimising water loss. It is thus surprising that fully stimulated tubules of *Tenebrio* can secrete fluid at up to 25 nl min^{-1}. To avoid possible dehydration and excessive loss of K^+ and Cl^- ions, a precisely controlled mechanism

to terminate diuresis must be in place. This certainly appeared to be true for tubules of *Tenebrio*. Paper 1 shows that secretion rates of *Tenebrio* tubules stimulated by Tenmo-DH_{37} are reduced by very low doses of endogenous Tenmo-ADFa or ADFb or exogenous cardioacceleratory peptide 2b (CAP_{2b}). These factors increase the intracellular cyclic GMP concentration (Quinlan et al., 1997; Eigenheer et al., 2002), and this antagonizes the action of cyclic AMP, possibly by the activation of cyclic AMP-selective phosphodiesterases (Quinlan et al., 1997).

II. Immunolocalization of endogenous diuretic and antidiuretic peptides

The ability of a diuretic or antidiuretic peptide to stimulate or inhibit primary urine formation *in vitro* does not necessarily indicate its function *in vivo*. An important criterion that must be met is that these peptides must be synthesized in neurosecretory cells and transported to neurohaemal structures from where they are released into the circulation (Coast 1996). For this reason the immunolocalization of the diuretic peptide, Tenmo-DH_{37} (paper 2) and the antidiuretic peptide, Tenmo-ADFb (paper 3) was investigated.

The staining pattern of Tenmo-DH_{37} is fairly similar to that found for CRF-related peptides in *Locusta migratoria* (Patel et al., 1994; Thompson et al., 1995) and *Manduca sexta* (Veenstra and Hagedorn, 1991; Chen et al., 1994). The results indicate that Tenmo-DH_{37} is primarily synthesized in neurosecretory cells located in the pars intercerebralis of the brain and in the abdominal ganglia. The neurosecretory cells in the brain transport their Tenmo-DH_{37} immunoreactive material to the corpora cardiaca, from where it is released. The bilaterally arranged neurosecretory cell bodies in each of the abdominal ganglia project processes laterally to their respective neurohaemal release sites. Like the corpora cardiaca these neurohaemal release sites are in direct contact with the surrounding haemolymph ensuring fast release and transport of the peptide to its site of action, the Malpighian tubules.

Numerous Tenmo-DH_{37} immunoreactive cells were found in the anterior midgut with fine nerve processes running over the surface of the entire midgut. The possibility exists that these cells are responsible for the recycling of fluid from midgut back to haemolymph by rendering the gut epithelium more permeable. This study shows that Tenmo-DH_{37} is synthesized in neuroendocrine tissue from where it is transported to

neurohaemal organs and therefore brings this peptide closer to being reclassified as a hormone.

The staining pattern for Tenmo-ADFb was not as clear (paper 3). This potent antidiuretic peptide (EC_{50} 240 pM), isolated from *Tenebrio* heads, was found in neurosecretory cells located in the cerebral lobes of the *Tenebrio* brain. Although no staining of the corpora cardiaca was seen, these cells projected axons that entered a plexus of 'blebby' staining neuroreactive material located throughout the depth of the brain, characteristic of a neurohaemal release site. No Tenmo-ADFb immunoreactive cell bodies were seen, and the immunoreactive material present in all the abdominal ganglia followed no clear staining pattern. Immunoreactive cells were, however, found in the connectives and in the median nerve situated between the connectives of the metathoracic ganglion and the first abdominal ganglion. ADFb is identical to the 13 C-terminal residues of *Tenebrio* putative cuticle protein 9.2 (TmPCP 9.2; Baernholdt and Andersen, 1998). Although the immunocytochemical study does not provide a clear pattern, the results strongly suggest that ADFb is a true neuropeptide, and not just a mere artifact of proteolysis of *Tenebrio* putative cuticle protein TmPCP 9.2.

III. *Cellular mechanisms of fluid secretion*

1. High K^+ permeability of the basolateral membrane

During fluid secretion by insect tubules K^+ or Na^+ ions are transported from the haemolymph to the lumen against a steep electrochemical gradient. Transport of these cations is achieved by an apical vacuolar-type H^+-ATPase that operates in parallel with a cation/H^+ exchanger (Wiezoreck et al., 1989, 1991). Ion substitution experiments (paper 4) show that the basolateral membrane potential (V_{bl}) of *Tenebrio* tubules is very sensitive to the bath K^+ concentration, but not to Na^+. Fluid secretion rates appear to be totally dependent on the K^+ concentration of the bath and tubules stopped secreting entirely when the bath K^+ concentration reached 5 mM K^+. Paper 4 further investigated the mechanisms utilized by K^+ ions to cross the basolateral membrane. A decrease in fluid secretion and hyperpolarization of V_{bl} seen in the presence of Ba^{2+} strongly suggests the existence of K^+ channels in the basolateral membrane of *Tenebrio* tubules and indicates a cell-inward electrochemical gradient

for K^+. Unlike those of *Formica*, tubules of *Tenebrio* do not lose their sensitivity to the surrounding K^+ concentration in the presence of Ba^{2+} (Weltens et al., 1992).

2. Other K^+ uptake mechanisms across the basolateral membrane

Basal K^+ channels are not the only K^+ entry mechanism. A considerable decrease in fluid secretion in the presence of bumetanide (10^{-5} M) suggests K^+ uptake *via* a $Na^+/K^+/2Cl$ cotransporter. Inhibitory effects of bumetanide or furosemide on fluid secretion rates have also been reported for Malpighian tubules of *Drosophila* (Wessing et al., 1987), *Rhodnius* (O'Donnell and Maddrell, 1984) and *Formica* (Leyssens et al., 1994). Tubules of *Formica* appear to tolerate wide variations in haemolymph K^+ concentration (Van Kerkhove et al., 1989) and coupled entry of K^+, Na^+ and Cl^- in *Formica via* the cotransporter only appeared to become important when the bath K^+ concentration dropped below 10 mM (Leyssens et al., 1994). Like Malpighian tubules of *Formica* (Leyssens et al., 1994) and *Onymacris* (Isaacson et al., 1989), *Tenebrio* tubules showed no significant changes in membrane potetial when bumetanide was present.

The apparent insensitivity to ouabain reported for some insect Malpighian tubules argues against a major role for a Na^+/K^+-ATPase in fluid secretion. However, active K^+ uptake *via* the Na^+/K^+ ATPase appeared to be important in Malpighian tubules of *Tenebrio*. Ouabain seriously impaired fluid secretion, yet had no immediate effect on the electrical potentials in control Ringer (50 mM K^+).

3. Evidence for K_{ATP} channels in the basolateral membrane

In transporting epithelia of vertebrates, inhibition of the Na^+/K^+-ATPase by ouabain increases the intracellular ATP concentration, which in turn reduces the open probability of ATP-regulated K^+ (K_{ATP}) channels (Balaban et al., 1980; Hurst et al., 1993; Urbach et al., 1996). The possibility of the existence of K_{ATP} channels in Malpighian tubules of *Tenebrio* was investigated after V_{bl} responded with a hyperpolarization to ouabain in the presence of Ba^{2+} and low bath K^+ (paper 5). Glibenclamide, a substance generally used to block K_{ATP} channels, irreversibly decreased tubule secretion rates and, depending on the electrochemical gradient for K^+, hyperpolarized or depolarized V_{bl} in Ba^{2+} and low bath K^+ (5 mM K^+). In addition, glibenclamide changed the sensitivity of the basolateral membrane, making

it less sensitive to the surrounding K^+ concentration. A depolarization of V_{bl} in response to ouabain was previously reported in Malpighian tubules of *Locusta* (Baldrick et al., 1988) and *Drosophila* (Wessing et al., 1987), but was only seen in *Tenebrio* tubule cells after pretreatment with glibenclamide. This data strongly suggests the existence of K_{ATP} channels in tubules of *Tenebrio* and is the first study to implicate K_{ATP} channels in an insect epithelium (paper 5).

4. Mode of action of endogenous diuretic and antidiuretic peptides

The endogenous diuretic hormone Tenmo-DH_{37} and its second messenger cyclic AMP increase fluid secretion rates of *Tenebrio* tubules by increasing transepithelial transport of K^+ and possibly Cl^-. However, an increase in K^+ conductance alone depolarizes the basolateral membrane, whereas a stimulatory effect on the pump would result in a hyperpolarization. Blocking the K^+ conductance with Ba^{2+} unmasks the pump current (Weltens et al., 1992) and in these conditions Tenmo-DH_{37} and cyclic AMP hyperpolarized V_{bl} and depolarized the transepithelial potential (V_{te}) with no significant change in V_{ap} (paper 6). This indirectly indicates an increase of K^+ conductance *via* K^+ channels and a stimulation of the apical pump current. Possibly the depolarization of V_{te} shows the increase of Cl^- conductance across the apical membrane, but confirmation of this is subject to more experiments. Bafilomycin A_1 reversed the hyperpolarization of V_{bl} with Tenmo-DH_{37} and significantly decreased fluid secretion rates of tubules stimulated with cyclic AMP, demonstrating the presence of an apical V-ATPase in the tubules of *Tenebrio*.

The endogenous antidiuretic peptide Tenmo-ADFa and its second messenger cyclic GMP had the opposite effect on the electrical profile, i.e. depolarization of V_{bl} and hyperpolarization of V_{te}, indicating a decrease in K^+ conductance across the basolateral membrane and an inhibitory effect on the apical proton pump. A similar response was reported for the ant antidiuretic factor Fop-ADF, for which the second messenger involved still requires characterization (Laenen et al., 2001).

In the presence of the $Na^+/K^+/2Cl^-$ cotransporter blocker, bumetanide, Tenmo-DH_{37} and cyclic AMP showed no visible effect on the basolateral membrane potential. Although the presence and the importance of the $Na^+/K^+/2Cl^-$ cotransporter in

Tenebrio tubule secretion have been established (see paper 4), the possible effect of Tenmo-DH_{37} and cyclic AMP on this transporter requires more investigation.

Finally, the effect of amiloride on fluid secretion rates, luminal pH and the electrical profile indicated the existence of a K^+/nH^+ antiporter in Malpighian tubules of *Tenebrio*.

Model for transepithelial K^+ transport

The electrophysiological data presented in this study made it possible to propose a model for transepithelial K^+ transport in Malpighian tubules of *Tenebrio* (Fig A). Driven by a favorable electrochemical gradient, basal K^+-uptake occurs *via* K^+ channels and a $Na^+/K^+/2Cl$ cotransporter. Active K^+ uptake *via* the Na^+/K^+-ATPase appears very important and uptake *via* K_{ATP} channels is particularly apparent at a low bath K^+ and high Na^+ concentration (5 mM K^+, 140 mM Na^+). Possibly the Na^+/K^+-ATPase activity is high and the local ATP concentration at the basolateral membrane is low at this bath concentration. At the apical membrane, K^+ extrusion into the lumen is achieved *via* a K^+/nH^+ antiporter in parallel with an electrogenic H^+ pump. Endogenous *Tenebrio* diuretic and antidiuretic peptides and their second messengers act on the K^+ transport *via* barium sensitive K^+ channels, the apical bafilomycin A_1 sensitive H^+ pump and possibly paracellular or transcellular Cl^- transport (Fig B).

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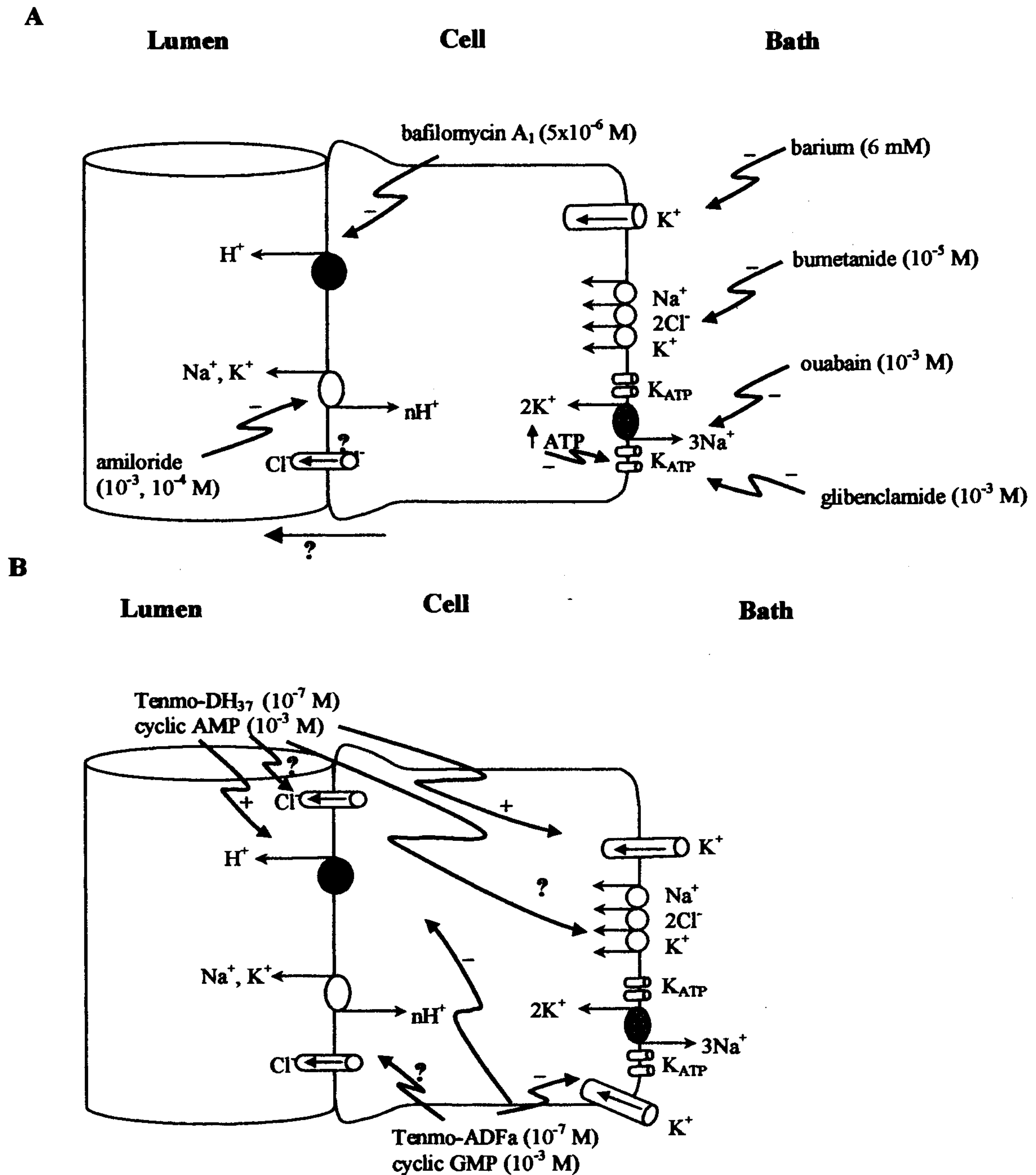

Fig. (A) Model for transepithelial K^+ transport in Malpighian tubules of *Tenebrio*. (B) Transport mechanisms influenced by endogenous *Tenebrio* diuretic and antidiuretic peptides and their second messengers. The effect of various inhibitors/stimulants applied from the bath side on the different transport systems is shown.

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