The Journal of Experimental Biology 211, 42-48
Published by The Company of Biologists 2008
doi:10.1242/jeb.011882

Differential actions of diuretic factors on the Malpighian tubules of *Rhodnius prolixus*

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Accepted 22 October 2007

SUMMARY

The effects of corticotropin-releasing factor (CRF)-related (ZooneDH), calcitonin (CT)-related ($RhoprDH_{31}$) and kinin-related (leucokinin I) peptides on the ion composition of fluid secreted by upper *Rhodnius prolixus* Malpighian tubules and on KCl reabsorption by the lower tubules were assessed. ZooneDH stimulated fluid secretion while increasing the [Na^+] of secreted fluid at the expense of [K^+]. Upper tubules responded to ZooneDH with a characteristic triphasic change in the transepithelial potential (TEP), reminiscent of the response to 5-hydroxytryptamine (5HT). $RhoprDH_{31}$ produced a small (~9 mV) lumen-positive shift in TEP of the upper tubule but had no effect on the rate of fluid secretion or ion composition of the secreted fluid. In contrast to 5HT, both peptides failed to activate KCl reabsorption by the lower tubule. Leucokinin I had no effect on the ion composition of fluid secreted by whole or upper Malpighian tubules. We propose that: (1) 5HT and a native CRF-related peptide similar to ZooneDH activate the same second messenger systems and ion transporters in the upper tubule cells; (2) CRF-related peptide is utilized to maintain high rates of fluid secretion during the post-feeding diuresis and is additionally used at times when KCl reabsorption is unnecessary or detrimental. The differential actions of multiple diuretic factors allows for intricate control of ionic and osmotic balance in *R. prolixus*.

Key words: *Rhodnius*, diuretic hormone, Malpighian tubules, transepithelial potential, secreted fluid, CRF-related peptide, kinin, DH31.

INTRODUCTION

Rhodnius prolixus periodically ingests a blood meal equivalent to 10 times its unfed weight. At these times, a rapid diuresis is initiated and the insect eliminates a large portion of the water and NaCl associated with the blood meal, while preserving its ionic and osmotic balance. The diuresis is controlled by the timely release of the hormone 5-hydroxytryptamine (5HT) and at least one neuropeptide, which is believed to also be a diuretic hormone (DH) (Lange et al., 1989; Maddrell et al., 1991; Maddrell, 1963). Both hormones stimulate rapid fluid secretion by the upper secretory segment of the Malpighian tubules (Maddrell, 1963; Maddrell et al., 1991). Stimulation by 5HT produces a characteristic triphasic change in transepithelial potential (TEP), corresponding to sequential activation of an apical Cl^- conductance (phase 1), an apical V-type H^+-ATPase (phase 2) and a basal Na^+–K^+–$2Cl^-$ cotransporter (phase 3) (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001). The resulting movement of Na^+, K^+ and Cl^- from the hemolymph, across the tubule cells and into the tubule lumen drives the flow of osmotically obliged water through aquaporin-like channels (Echevarria et al., 2001; Martini et al., 2004). The actions of 5HT on the upper tubule are mediated by the second messenger cyclic AMP (Maddrell et al., 1971; Montoreano et al., 1990; Martini et al., 2004). Depletion of K^+ from the hemolymph during this rapid diuresis is prevented by KCl reabsorbtion by the tubule's lower segment, through processes that are also stimulated by 5HT (Maddrell, 1978; Maddrell et al., 1993; Haley and O'Donnell, 1997). The effect of the peptidergic DH on the fluid secretion rates of upper tubules is similar to that of 5HT, but studies on the exact mechanisms of its actions have been hindered because the hormone has yet to be identified.

A number of peptides with diuretic effects have been discovered in other insects. These peptides belong to one of at least three families of peptides; the corticotropin-releasing factor (CRF)-related DHs, the calcitonin (CT)-related DHs and the kinin-related DHs. The CRF- and CT-related DHs act through cyclic AMP in tubules of several species to activate various transporters like the V-type H^+-ATPase, Na^+ channels and the Na^+–K^+–$2Cl^-$ cotransporter (Plawner et al., 1991; Coast et al., 2005; Coast et al., 2002; Cabrero et al., 2002; Coast et al., 2001). The kinin-related DHs generally act through an increase in intracellular Ca^{2+} levels to activate Cl^- conductance pathways (O'Donnell et al., 1998; Beyenbach, 2003). The presence and release into the hemolymph of CRF-related, CT-related and kinin-related peptides in *R. prolixus* have been suggested on the basis of immunohistochemical, radioimmunological and high performance liquid chromatographic techniques (Te Brugge et al., 1999; Te Brugge and Orchard, 2002; Te Brugge et al., 2005). The exogenous CRF-related peptides ZooneDH and $DippuDH_{46}$ from *Zootermopsis nevadensis* and *Diploptera punctata*, respectively, stimulate fluid secretion and increase the cyclic AMP content of the upper tubules of *R. prolixus* (Te Brugge et al., 2002). The CRF-related peptides are potent stimulators of fluid secretion, capable of eliciting maximal rates of secretion comparable to those of 5HT-stimulated tubules (Te Brugge et al., 2002). In contrast, the CT-related peptide $DippuDH_{31}$ produces only small increases in secretion rates of *R. prolixus* upper

tubules, equivalent to ~1.5% of maximal rates achieved with 5HT (Te Brugge et al., 2005). Interestingly, the CT-related peptide of *R. prolixus* has an identical sequence to that of $DippuDH_{31}$ and is herein referred to as $RhoprDH_{31}$ (Te Brugge et al., in press). The non-native kinin-related peptides leucokinin I, leucokinin VIII and locustakinin all fail to increase the rate of fluid secretion in *R. prolixus* tubules (Te Brugge et al., 2002).

To date, no studies have been performed on the effects of CRF-related, CT-related and kinin-related peptides on TEP or the ion composition of the fluid secreted by the upper tubules. Furthermore, it is not known whether these factors activate KCl reabsorption by the lower tubule. The present study reports the K^+, Na^+ and Cl^- concentrations of the secreted fluid from upper tubules before and after treatment with 5HT, ZooneDH, $RhoprDH_{31}$ and leucokinin I. The results demonstrate that the actions of ZooneDH on the upper tubules are similar to those of 5HT. In addition, none of the peptides were capable of activating KCl reabsorption by the lower tubule.

MATERIALS AND METHODS

Animals

Individuals of *Rhodnius prolixus* (Stål 1859) were obtained from a laboratory colony fed on rabbit blood and housed at 25°C under high humidity at the University of Toronto. Studies were conducted on unfed 5th instar larvae, 6–8 weeks post-emergence and that had previously fed on rabbit's blood as 4th instars. Experiments were conducted at room temperature (~20°C).

Upper Malpighian tubule secretion assay

Insects were dissected under saline that contained (mmol l^{-1}): 129 NaCl, 8.6 KCl, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 8.5 $MgCl_2$, 2 $CaCl_2$, 8.6 Hepes and 20 glucose at pH 7. Fine glass probes were used to dissect individual whole Malpighian tubules from the insects and the tubules were cut at the junction of the upper and lower segments. Individual upper segments of the tubules were transferred into 90 μl droplets of saline in a Sylgard-lined dish held under water-saturated paraffin oil. The open end of each tubule was pulled out of the saline droplet and wrapped around a minuten pin stuck into the Sylgard. Tubules were initially stimulated by adding a final concentration of 25 nmol l^{-1} 5HT to the saline droplets since unstimulated tubules secrete at very low rates of about 0.1 nl min^{-1}. ZooneDH, $RhoprDH_{31}$ and leucokinin I were subsequently added to the saline droplet at the final concentrations noted in the Results. Either for comparison purposes or to serve as a positive control, tubules in some experiments were treated with 1 μmol l^{-1} 5HT. Droplets of secreted fluid that formed at the pin were collected at intervals using fine glass probes. Fluid secretion rates were determined by measuring the diameter of the secreted fluid droplets with an ocular micrometer, calculating their volumes as $(\pi d^3)/6$, where d is the diameter, and dividing the volume by the time over which the droplets formed. The Na^+, K^+ and Cl^- concentrations of secreted fluid droplets were measured using ion-selective microelectrodes, as described below.

Whole Malpighian tubule secretion assay

The procedure for the dissection of whole tubules was the same as that described for the upper Malpighian tubules with the exception that the terminal ampulla was included and the tubules were not cut at the junction of the upper and lower segments. Whole tubules were transferred to saline droplets held under water-saturated paraffin oil. Each tubule was arranged such that the upper segment was held in a 90 μl droplet of saline modified to contain 24 mmol l^{-1} KCl and 113.6 mmol l^{-1} NaCl (all other solutes unaltered) and the lower segment was held in a separate 90 μl droplet of saline containing 4 mmol l^{-1} KCl and 133.6 mmol l^{-1} NaCl. This approach has been shown to maintain high rates of fluid and K^+ secretion by the upper tubule and high rates of KCl reabsorption by the lower tubule (Haley and O'Donnell, 1997; Maddrell et al., 1993). The junction of the upper and lower segments was positioned in the paraffin oil between the two saline droplets. The ampulla was pulled out of the droplet holding the lower segment of the tubule and wrapped around a minuten pin. The upper segments of the tubules were stimulated by 1 μmol l^{-1} 5HT. Droplets of secreted fluid that had passed through the lower segment and emerged at the ampulla were collected for 10–15 min. This was followed by the addition of 1 μmol l^{-1} ZooneDH, 0.1 μmol l^{-1} $RhoprDH_{31}$ or 0.1 μmol l^{-1} leucokinin I, the same concentrations of all three peptides at once, or 1 mmol l^{-1} 8-bromo-cyclic AMP to the saline droplet containing the lower segment of the tubule, and collection of fluid for a further 20–30 min. 5HT (1 μmol l^{-1}) was then added to the droplet bathing the lower tubule and fluid was collected for 10–30 min. Lastly, the tubule was cut at the junction between the upper and lower segments and fluid emerging from the upper segment was collected. Ion concentrations in the collected droplets were measured using ion-selective microelectrodes.

Measurement of the transepithelial potential of upper Malpighian tubules

A previous study has validated the use of the Ramsay technique for the measurement of TEP in upper Malpighian tubules of *R. prolixus* (Ianowski and O'Donnell, 2001). Isolated upper tubules from 5th instars were transferred to saline droplets under paraffin oil. The cut end of the tubule was pulled out of the droplet and wrapped around a minuten pin. A fine glass probe was used to make a small hole in the tubule wall between the saline droplet and the minuten pin. The tip of a microelectrode filled with 3 mol l^{-1} KCl was placed in the secreted fluid that emerged from the hole in the tubule while a second, similar microelectrode was placed in the saline droplet. The electrodes were connected through a high impedance (10^{13} Ω) ML165 pH Amp to a Powerlab 4/30 data acquisition system (ADInstruments, Colorado Springs, CO, USA). ZooneDH (1 μmol l^{-1}) or $RhoprDH_{31}$ (0.1 μmol l^{-1}) was added to the saline droplet while recording the transepithelial potential.

Construction of ion-selective microelectrodes

Microelectrodes were fabricated as described previously (Rheault and O'Donnell, 2001; Donini and O'Donnell, 2005). The following ionophore cocktails and backfill solutions (in parentheses) were used: Na^+ ionophore II cocktail A (500 mmol l^{-1} NaCl), K^+ ionophore I cocktail B (500 mmol l^{-1} KCl). The tips of these electrodes were dipped in a solution of polyvinylchloride (PVC, Fluka, Buchs, Switzerland) in tetrahydrofuran (THF, Fluka) to permit their use in fluid droplets under paraffin oil (see Rheault and O'Donnell, 2004). For the measurement of Cl^-, a solid state silver/silver chloride microelectrode was employed as previously described (Donini and O'Donnell, 2005). The microelectrodes were calibrated in the following solutions: Na^+, 15 mmol l^{-1} NaCl/135 mmol $^{-1}$ LiCl and 150 mmol l^{-1} NaCl; K^+, 15 mmol l^{-1} KCl/135 mmol l^{-1} NaCl and 150 mmol l^{-1} KCl; Cl^-, 20 mmol l^{-1} KCl and 200 mmol l^{-1} KCl. Slopes for the electrodes [mean ± s.e.m. (*N*)] for a tenfold change in ion concentration were 53.9±1.4 mV (9) for Na^+, 55.1±0.5 mV (26) for K^+ and 58.2±2 mV (9) for Cl^-. The reference microelectrode was filled with a solution

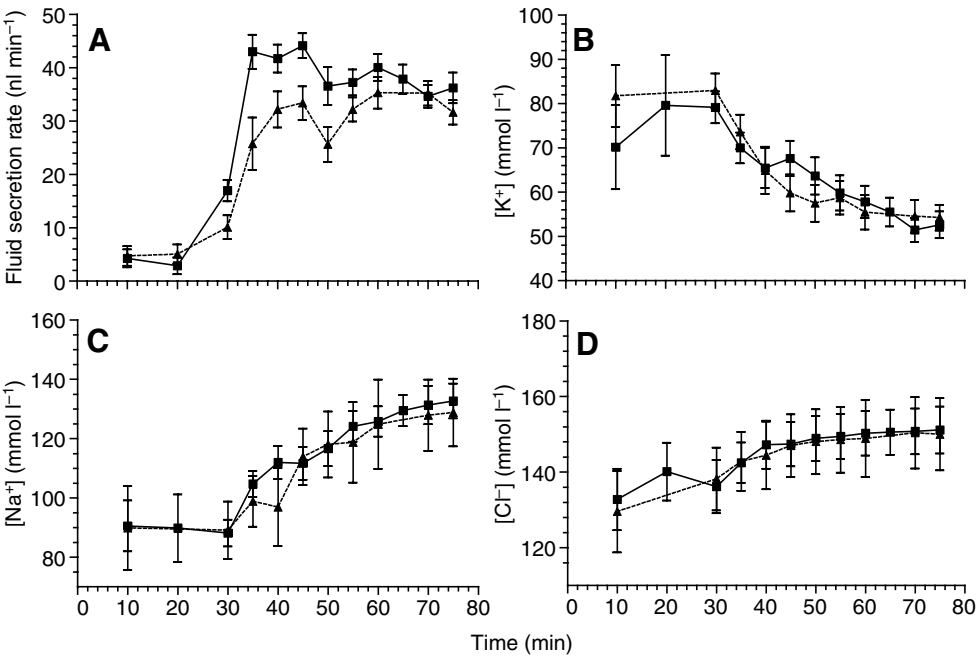

Fig. 1. Upper tubules were initially stimulated with 25 nmol l^{-1} 5-hydroxytryptamine (5HT) in saline and fluid was collected at 10 min intervals for a total of 20 min. The tubules then received 1 μmol l^{-1} of either 5HT (filled squares) or ZooneDH (filled triangles) and fluid was collected for a further 55 min at 5 min intervals. The rate of fluid secretion (A) was calculated after measuring the diameter of each droplet, and ion-selective microelectrodes were used to measure the K^+ (B), Na^+ (C) and Cl^- (D) concentration of each droplet of secreted fluid. ZooneDH and 5HT stimulate fluid secretion to a similar extent and both increase the Na^+ concentration of the secreted fluid at the expense of K^+ over time. Each point is the mean ± s.e.m. of 6–9 individual tubules.

of 500 mmol l^{-1} KCl. Electrodes were connected to a data acquisition system as described for the measurement of transepithelial potential. The ion concentration of the secreted fluid was calculated using the following formula: $[ion]_{sf}=C\times10^{(\Delta V/slope)}$, where $[ion]_{sf}$ is the ion concentration in the secreted fluid droplet; C is the ion concentration in one of the calibration solutions used to calibrate the electrodes; ΔV is the voltage difference between the droplet of secreted fluid and the same calibration solution; and the slope is the change in voltage measured by the electrode in response to a tenfold change in ion concentration. K^+ interferes with the response of the Na^+ electrode, and the Na^+ concentrations were therefore corrected using the Nicolsky–Eisenman equation (Ammann, 1986) and a selectivity coefficient (K_{NaK}) of $10^{0.32}$. Although ion-selective microelectrodes measure ion activity and not concentration, data can be expressed in terms of concentration if it is assumed that the ion activity coefficient is the same in calibration and experimental solutions.

RESULTS

Fluid secretion rates and secreted fluid ion composition for the upper tubule

The actions of 5HT on the upper, secretory segment of the Malpighian tubules of *R. prolixus* have been well documented (O'Donnell and Maddrell, 1984; Maddrell et al., 1991; Ianowski and O'Donnell, 2001). Fluid secreted by unstimulated upper tubules is K^+ rich, whereas that secreted by 5HT-stimulated tubules contains approximately equal concentrations of Na^+ and K^+ (Maddrell and Overton, 1988). Equimolar amounts of Na^+ and K^+ were evident when the upper tubules were partially stimulated by a low concentration (25 nmol l^{-1}) of 5HT, and secreting at rates of approximately 5 nl min^{-1}, equivalent to ~15% of the maximal rate (Fig. 1A–C). For tubules that subsequently received (1 μmol l^{-1}) 5HT or ZooneDH the $[K^+]/[Na^+]$ ratios in response to 25 nmol l^{-1} 5HT were [mean ± s.e.m. (N)] 0.83±0.14 (6) and 1.03±0.15 (7), respectively.

Addition of 5HT (1 μmol l^{-1}) produced a rapid increase in the rate of fluid secretion, which peaked within 15 min (Fig. 1A). ZooneDH (1 μmol l^{-1}) produced a similar increase in the rate of fluid secretion, although the response was slower, peaking at 25 min after applying ZooneDH.

Both 5HT and ZooneDH (1 μmol l^{-1}) caused an increase in the Na^+ concentration of the secreted fluid at the expense of K^+ over time (Fig. 1B,C). The Na^+ and K^+ concentration of the secreted fluid changed by approximately 20–30 mmol l^{-1}. The $[K^+]/[Na^+]$ ratios 55 min after the addition of 1 μmol l^{-1} 5HT or ZooneDH were 0.41±0.03 and 0.44±0.04, respectively. These values are significantly lower than those in response to 25 nmol l^{-1} 5HT alone (Student's t-test, P=0.003 and P=0.001 for 5HT and ZooneDH, respectively). Neither 5HT nor ZooneDH had significant effects on the Cl^- concentration of the secreted fluid over time (Fig. 1D).

R. prolixus upper tubules are sensitive to changes in the bath concentration of K^+, responding to a decrease in K^+ bath concentration with a decrease in the overall secretion of K^+ (Maddrell et al., 1993). However, our results do not reflect K^+ depletion of the bathing saline resulting from the high rates of ion transport by the tubules. The final $[K^+]$ in the bathing droplet at the end of the experiments (9.78±0.7 mmol l^{-1}) was not different from that at the beginning (8.45±0.3 mmol l^{-1}) for tubules stimulated with 5HT. Corresponding values for tubules stimulated with ZooneDH were 8.1±0.6 and 9.1±0.2 mmol l^{-1}, respectively.

A high concentration (0.1 μmol l^{-1}) of $RhoprDH_{31}$ alone increases fluid secretion by upper tubules to 1.5% of the maximal value seen in response to 1 μmol l^{-1} 5HT (Te Brugge et al., 2005). There is no effect of leucokinin I (0.1 μmol l^{-1}) on fluid secretion rate (Te Brugge et al., 2005). In the presence of a low concentration of 5HT (25 nmol l^{-1}), neither $RhoprDH_{31}$ nor leucokinin I (0.1 μmol l^{-1}) altered fluid secretion rate or the composition of the secreted fluid (Fig. 2). These tubules secreted at high rates upon the addition of 1 μmol l^{-1} 5HT over the final 15 min of the experiment and without altering the $[K^+]/[Na^+]$ ratio.

Transepithelial potential of the upper tubule

The effect of a high concentration of 5HT on the TEP of the upper tubules has been described in detail (O'Donnell and Maddrell, 1984; Ianowski and O'Donnell, 2001) and consists of a characteristic triphasic response. Unstimulated tubules have a

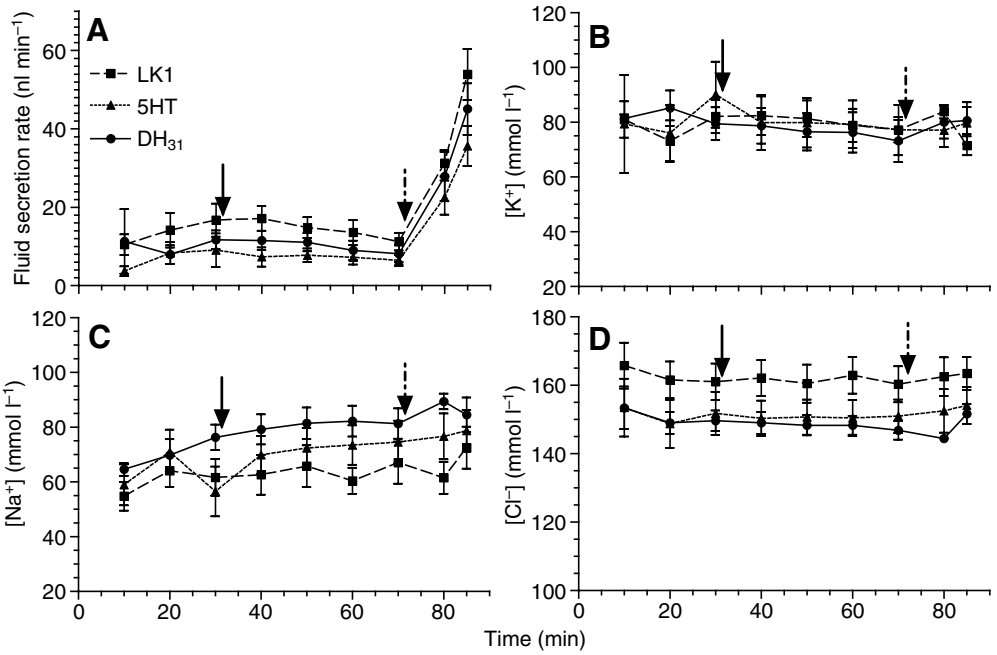

Fig. 2. Upper tubules were initially stimulated with 25 nmol l^{-1} 5HT in saline and fluid was collected at 10 min intervals for a total of 30 min. The tubules were then maintained in 25 nmol l^{-1} 5HT (filled triangles) or received 0.1 μmol l^{-1} of either $RhoprDH_{31}$ (filled circles) or leucokinin I (filled squares) at the solid arrow, and fluid was collected for a further 40 min at 10 min intervals. The rate of fluid secretion (A) was calculated after measuring the diameter of each droplet, and ion-selective microelectrodes were used to measure the K^+ (B), Na^+ (C) and Cl^- (D) concentration of each droplet of secreted fluid. $RhoprDH_{31}$ and leucokinin I had no effect on the rate of fluid secretion or the ion composition of the secreted fluid. Each point is the mean ± s.e.m. of 7–9 individual tubules. Addition of 1 μmol l^{-1} 5HT at the dashed arrow was used to confirm the viability of the Malpighian tubules.

lumen-negative TEP and application of a high concentration of ZooneDH caused the TEP to change in three phases, similar to the 5HT response (Fig. 3). The initial phase occurred within 1 min of ZooneDH application and was characterized by a negative deflection of the TEP from a value of –24.6±6.2 mV to –29.2±6.1 mV (*N*=5). Phase 2 peaked within 5 min of ZooneDH application and caused a large positive deflection of the TEP to a value of +20.2±8 mV. In phase 3, the TEP returned to a value of –19.1±8.9 mV, which was similar to but more positive than the value in unstimulated tubules.

A high concentration of $RhoprDH_{31}$ resulted in a positive deflection of the TEP from the unstimulated value of –34.8±3.8 mV to –25±3.4 mV (*N*=5), which stabilized within 5 min. These tubules subsequently responded to a high concentration of 5HT in the characteristic triphasic manner with phase 1 consisting of a slight (2 mV) negative deflection, phase 2 a large (60 mV) positive deflection and phase 3 returning to –15±5.3 mV, almost 20 mV more positive than the TEP prior to the addition of 5HT.

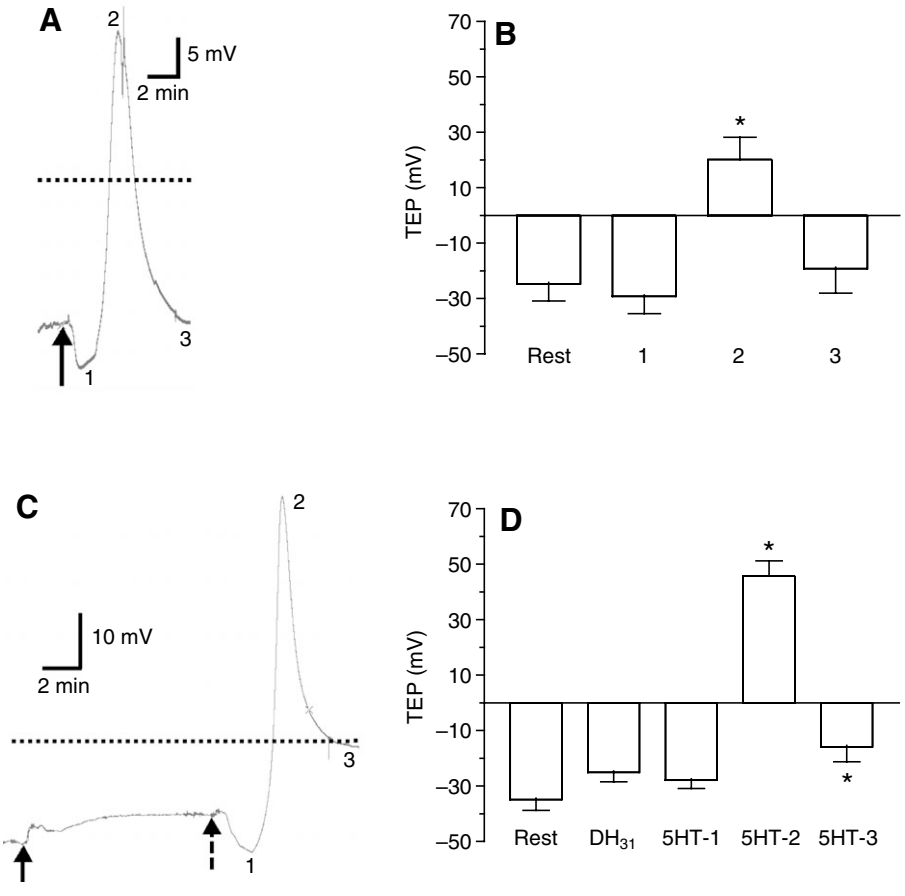

Fig. 3. Transepithelial potential (TEP) of the upper, secretory segment of the Malpighian tubules. (A) Representative recording showing the effects of 1 μmol l^{-1} ZooneDH (arrow). Three distinct phases of the response are numbered on the trace. The dotted line indicates a potential of 0 mV, when both microelectrodes were placed in the bathing droplet. (B) The mean ± s.e.m. (*N*=5) TEP prior to the addition of ZooneDH (Rest) and during the three phases of the response. (C) Representative recording showing the effects of 0.1 μmol l^{-1} $RhoprDH_{31}$ (solid arrow) and 1 μmol l^{-1} 5HT (dashed arrow). The three phases of the TEP response to 5HT are numbered. (D) The mean ± s.e.m. (*N*=5) TEP prior to the addition of $RhoprDH_{31}$ (Rest), 10 min after the addition of $RhoprDH_{31}$ (DH_{31}), and during the three phases of the 5HT response. Asterisks denote a significant difference from the resting TEP (ANOVA, followed by Tukey–Kramer).

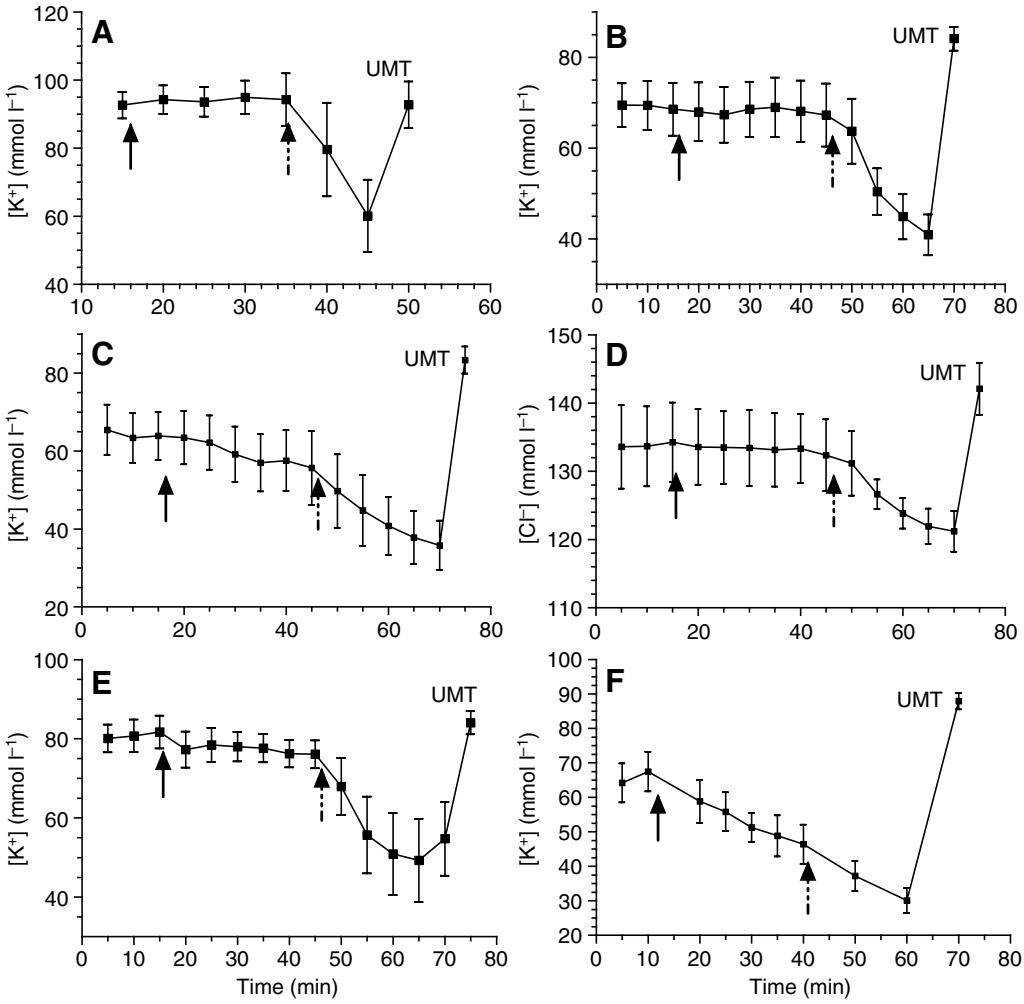

Fig. 4. Whole Malpighian tubules were removed from animals and a modified Ramsay secretion assay employing two separate bathing droplets for the upper and lower segments of the tubules was used to collect secreted fluid. Ion-selective microelectrodes were used to measure the K^+ and Cl^- concentration of the secreted fluid. Upper tubules were stimulated with 1 μmol l^{-1} 5HT and the lower tubules received 1 μmol l^{-1} ZooneDH (A), 0.1 μmol l^{-1} $RhoprDH_{31}$ (B), 0.1 μmol l^{-1} leucokinin I (C,D), all three peptides at once (E) or 1 mmol l^{-1} 8-bromo-cyclic AMP (F; solid arrow in A–F). In addition, the lower tubules subsequently received 1 μmol l^{-1} 5HT (dashed arrow in A–F) as a positive control. A sample of the fluid from the upper tubule was taken at the end of the experiment by cutting the tubule at the junction of the upper and lower tubule (UMT). Values are means ± s.e.m. for A: *N*=4; B: *N*=5; C and D: *N*=6–11; E: *N*=7; F: *N*=6.

KCl reabsorption by the lower tubule

The lower segment of the *R. prolixus* Malpighian tubule reabsorbs KCl when stimulated with 5HT (Maddrell et al., 1993). The K^+ concentration of the secreted fluid collected from whole tubules was unaffected by ZooneDH, $RhoprDH_{31}$ or leucokinin I applied individually (Fig. 4A–C) or in unison (Fig. 4E). We also examined the Cl^- concentration of the fluid secreted by whole tubules in response to leucokinin I because of the effects of this peptide on Cl^- conductance in tubules of other species. Leucokinin I had no effect on the Cl^- concentration (Fig. 4D). In all cases, the tubules were capable of reabsorbing K^+ as seen by the response to 5HT near the end of each experiment (Fig. 4). Since 5HT is known to act through the second messenger cyclic AMP in the upper tubule, we assessed the effects of the membrane-permeable and phosphodiesterase-resistant cyclic AMP analog 8-bromo-cyclic AMP on the lower tubule. There was a partial stimulation of K^+ reabsorption as seen by the decrease in K^+ concentration of the fluid secreted by whole tubules (Fig. 4F).

DISCUSSION

This is the first study to examine the actions of CRF-related, CT-related and kinin-related peptides on the ion composition of secreted fluid and/or on transepithelial potentials of Malpighian tubules of *R. prolixus*. The CRF-related peptides ZooneDH, $DippuDH_{46}$ and LocustaDH were previously shown to stimulate the fluid secretion of *R. prolixus* Malpighian tubules at rates similar to those elicited by high concentrations of 5HT (Te Brugge et al., 2002; Coast, 1997). Upon application of ZooneDH to upper segments of the tubules and in the presence of a low concentration of 5HT, the Na^+ concentration of the secreted fluid increased at the expense of K^+. This effect was similar to the effect of a high concentration of 5HT on the tubules. In both cases the $[K^+]/[Na^+]$ ratio was significantly reduced over time. The resulting K^+ and Na^+ concentrations of the secreted fluid collected 55 min after application of 1 μmol l^{-1} 5HT were 52±3 and 132±5.8 mmol l^{-1}, respectively. The K^+ concentration is slightly lower than the 70 mmol l^{-1} previously reported by Maddrell and Phillips (Maddrell and Phillips, 1975), whereas the Na^+ concentration is similar to the value of 125 mmol l^{-1} reported in the same study. The apparent natriuretic activity of ZooneDH is consistent with another CRF-related and native peptide, Achdo-DH, in the cricket (Coast et al., 2002); however, another CRF-related peptide in the mosquito, $Anoga\text{-}DH_{44}$, does not possess natriuretic activity (Coast et al., 2005). All these peptides, as well as 5HT in *R. prolixus*, appear to act through cyclic AMP.

Detailed comparison of the changes in transepithelial potential in response to ZooneDH and 5HT reveals that the magnitude and timing of each of the three phases is approximately the same. Pharmacological studies have attributed each phase of the 5HT response to a specific ion-transport mechanism in the apical or basal membranes of the upper tubule cells (Ianowski and O'Donnell, 2001). It therefore seems likely that ZooneDH affects the same ion

transporters as 5HT. Our results, coupled with previous findings that ZooneDH and 5HT both increase intracellular cyclic AMP levels (see Te Brugge et al., 1999; Te Brugge et al., 2002) in the upper tubule cells, strongly suggest that a native CRF-related peptide and 5HT in *R. prolixus* activate the same second messenger pathways and ion-transport mechanisms in the upper tubule cells.

In our studies, the native CT-related peptide $RhoprDH_{31}$ had no significant effect on the rate of fluid secretion or the ion composition of the secreted fluid when it was applied in the presence of 25 nmol l^{-1} 5HT. Previous reports showed a significant increase in the rate of fluid secretion when the peptide (referred to then as Dippu-DH_{31}) was applied alone; however, the maximal rates achieved were only ~1.5% of those obtained with a high concentration of 5HT, and the actions of the peptide were highly variable (Te Brugge et al., 2005). Given the small lumen-positive change in TEP in response to $RhoprDH_{31}$, the peptide may stimulate the V-type H^+-ATPase on the apical membrane (i.e. phase 2 of the response to 5HT or ZooneDH). If this is the case, then it would seem that the effects of $RhoprDH_{31}$ on the proton pump are relatively small (when compared with ZooneDH or 5HT). Co-localisation of 5HT and $RhoprDH_{31}$-like material is found in cell bodies and their neurohemal sites on the abdominal nerves, suggesting that these two factors may be co-released (Te Brugge et al., 2005). When 1 μmol l^{-1} 5HT is applied in the presence of $RhoprDH_{31}$, phase 1 of the 5HT response, which is proposed to correspond to activation of an apical Cl^- conductance, is reduced [~2 mV shift, see Fig. 3, and ~10 mV shift with 5HT alone (see Ianowski and O'Donnell, 2001)]. This pattern may reflect partial stimulation of proton pump activity by $RhoprDH_{31}$ and a consequent lumen-positive shift in TEP, so that the lumen-negative shift produced by the increase in Cl^- conductance during phase 1 of the 5HT response is reduced. Phase 2 of the 5HT response is no different in the presence of $RhoprDH_{31}$ (~60 mV shift from phase 1), which is consistent with a maximal stimulation of the proton pump. Phase 3 of the 5HT response, corresponding to the subsequent activation of the basal Na^+–K^+–2Cl^- cotransporter, in the presence of $RhoprDH_{31}$ resulted in a TEP which was ~20 mV more positive than the TEP prior to the addition of 5HT. In contrast, 5HT alone results in a phase 3 TEP, which is only ~10 mV more positive than the resting TEP (see Ianowski and O'Donnell, 2001) (Fig. 2A). This result is also consistent with a continued potentiation of proton pump activity by $RhoprDH_{31}$.

In contrast to the secretory nature of the upper tubules, the lowermost segments of the tubules are responsible for the reabsorption of KCl (Maddrell and Phillips, 1975). Reabsorption is activated by 5HT and the cells of the lower tubules respond more rapidly to 5HT than those of the upper tubule (Maddrell et al., 1993). This is a particularly important physiological role since the high rate of KCl transport from hemolymph to lumen by the upper tubules would quickly deplete the hemolymph of K^+ without reabsorption of K^+ downstream. Our results demonstrate that ZooneDH and $RhoprDH_{31}$ cannot activate KCl reabsorption by the lower tubules. Interestingly, Maddrell (Maddrell, 1976) suggested that different regions of the tubule are not controlled by a single hormone since extracts of lateral neurosecretory cells of the mesothoracic ganglionic mass stimulated secretion from the upper tubules but did not stimulate KCl reabsorption from the lower tubule. The membrane-permeable and phosphodiesterase-resistant cyclic AMP analog 8-bromo-cyclic AMP at least partially activates K^+ reabsorption, suggesting that cyclic AMP may act as a second messenger for activation of one or more of the KCl transporters in the lower tubule. Moreover, since 5HT, CRF-related and CT-related peptides all appear to make use of cyclic AMP as a second messenger, our results suggest that the cells of the lower tubules may not express receptors for CRF-related and CT-related peptides.

Leucokinin I had no measurable effect on the Malpighian tubules of *R. prolixus*. Te Brugge et al. (Te Brugge et al., 2002) tested for effects of a number of kinin-like peptides on the upper tubules of *R. prolixus*, revealing that none of these peptides affected the rate of fluid secretion. Based on our results, these findings can be extended to include no effect on the ion composition of the secreted fluid or on the lower tubule. In addition, application of all three peptides at once to the lower tubule had no effect on K^+ concentration of the secreted fluid from whole tubules.

In summary, this study demonstrates that various putative diuretic factors have different actions on the Malpighian tubules of *R. prolixus*. Although CRF-related factors and 5HT have similar, if not identical, actions on the upper tubules, an important difference is that CRF-related factors cannot activate KCl reabsorption by the lower tubule. The native CT-related factor $RhoprDH_{31}$ has minimal and variable effects on fluid secretion (Te Brugge et al., 2005) and produces a small (~9 mV) increase in the TEP of upper tubules. Although our findings suggest that kinin-like factors do not act on upper or lower Malpighian tubules of *R. prolixus*, kinins may still be important in the post-feeding diuresis by acting on other relevant tissues such as the crop and hindgut (see Te Brugge et al., 2002).

It thus appears that *R. prolixus* has multiple signaling mechanisms that permit complex control of hemolymph ionic and osmotic balance. During the post-feeding diuresis, we propose that the quick and timely release of 5HT (see Lange et al., 1989) serves to activate KCl reabsorption by the lower tubule and rapid fluid secretion by the upper tubule, which may be potentiated by the co-release of $RhoprDH_{31}$ (see Maddrell et al., 1993; Te Brugge et al., 2005). When hemolymph titers of 5HT fall to levels that are insufficient to maintain high rates of fluid secretion by the upper tubules, release of a native CRF-related peptide can be utilized to maintain these high rates of secretion. In addition there are likely to be other times when stimulation of upper tubule ion transport is needed without reabsorption of KCl by the lower tubule. For instance, digestion of the red blood cells from the bloodmeal may lead to a K^+ load, which the insect needs to eliminate. In this case the CRF-related peptide may be released in the absence of 5HT.

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