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THE *DROSOPHILA MELANOGASTER* HOMOLOGUE OF AN INSECT CALCITONIN-LIKE DIURETIC PEPTIDE STIMULATES V-ATPase ACTIVITY IN FRUIT FLY MALPIGHIAN TUBULES

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Summary

The *Drosophila melanogaster* homologue of an insect calcitonin-like diuretic hormone was identified in a BLAST search of the *Drosophila* genome database. The predicted 31-residue amidated peptide (*D. melanogaster* DH_{31}; Drome-DH_{31}) was synthesised and tested for activity on fruit fly Malpighian tubules. It increases tubule secretion by approximately 35 % of the response obtained with a myokinin from the housefly *Musca domestica* (muscakinin; Musdo-K) and has an EC_{50} of 4.3 nmol l^{-1}.

The diuretic activities of Drome-DH_{31} and Musdo-K were additive when tested at threshold and supra-maximal concentrations, which suggests that they target different transport processes. In support of this, Drome-DH_{31} increased the rate of secretion by tubules held in bathing fluid with a reduced Cl^- concentration, whereas Musdo-K did so only in the presence of Drome-DH_{31}. Stimulation with Drome-DH_{31} increased the lumen-positive transepithelial potential in the main secretory segment of the tubule. This was attributed to activation of an apical electrogenic proton-translocating V-ATPase in principal cells, since it was associated with hyperpolarisation of the apical membrane potential and acidification of secreted urine by 0.25 pH units.

Exogenous 8-bromo-cyclic AMP and cyclic GMP increased tubule secretion to the same extent as Drome-DH_{31} and, when tested together with the diuretic peptide, their activities were not additive. Stimulation with Drome-DH_{31} resulted in a dose-dependent increase in cyclic AMP production by tubules incubated in saline containing 0.5 mmol l^{-1} 3-isobutyl-1-methylxanthine, whereas cyclic GMP production was unchanged. Taken together, the data are consistent with Drome-DH_{31} activating an apical membrane V-ATPase *via* cyclic AMP. Since the K^+ concentration of the secreted urine was unchanged, it is likely that Drome-DH_{31} has an equal effect on K^+ and Na^+ entry across the basolateral membrane.

Key words: *Drosophila melanogaster*, Malpighian tubule, fluid secretion, diuretic hormone, calcitonin-like diuretic peptide, muscakinin, V-ATPase, cyclic AMP.

Introduction

Recently, Furuya et al. (Furuya et al., 2000) described the isolation of two diuretic hormones (DHs) from the corpora cardiaca of the Pacific beetle cockroach *Diploptera punctata*. The shorter peptide (31 amino acid residues) was named *D. punctata* DH_{31} (Dippu-DH_{31} following the species abbreviation used by NCBI/Swiss-Prot), while the longer peptide (46 residues) was named *D. punctata* DH_{46} (Dippu-DH_{46}). Both were identified on the basis of their ability to increase cyclic AMP production by Malpighian tubules of either the tobacco hornworm *Manduca sexta* (Dippu-DH_{46}) or the grasshopper *Schistocerca americana* (Dippu-DH_{31}), assays used previously for the identification of insect corticotropin (CRF)-related diuretic peptides (Kay et al., 1991a). The diuretic activity of each peptide was subsequently confirmed in a fluid secretion assay using Malpighian tubules from *Diploptera punctata* and the migratory locust *Locusta migratoria*. Dippu-DH_{46} belongs to the insect CRF-related peptide family (Schooley, 1993) and has 78 % sequence identity to a previously described peptide from *Periplaneta americana* (Peram-DP; Kay et al., 1992), whereas Dippu-DH_{31} is a novel peptide with some similarity to vertebrate calcitonin. The sequence similarity between Dippu-DH_{31} and calcitonin is small (seven residues are identical to calcitonins from chicken, eel and goldfish), but chicken calcitonin does stimulate fluid secretion by *Diploptera punctata* tubules (Furuya et al., 2000).

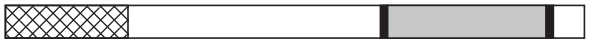
Although isolated using the same bioassay, Dippu-DH_{31} and Dippu-DH_{46} differ in their mode of action. The maximal diuretic activity of Dippu-DH_{31} is approximately 40 % that of Dippu-DH_{46}, and when tested together at sub-maximal

concentrations on cockroach tubules the combined response is greater than the sum of their separate activities, which provides evidence for synergism and indicates that they differ in their mode of action. Using Malpighian tubules of *Locusta migratoria*, it was possible to compare the activity of Dippu-DH_{31} with that of *Locusta*-DH (Locmi-DH; Lehmberg et al., 1991; Kay et al., 1991b) and locustakinin (Locmi-K; Schoofs et al., 1992). The actions of Dippu-DH_{31} resembled those of Locmi-K in that it acted synergistically with Locmi-DH and had no effect on the $[K^+]$:$[Na^+]$ ratio of the secreted urine. However, Dippu-DH_{31} also acts synergistically with Locmi-K, indicating a difference in their mode of action. Indeed, although Dippu-DH_{31} stimulates cyclic AMP production by *Schistocerca americana* tubules, Locmi-K has no effect on adenylyl cyclase activity in *Locusta migratoria* tubules (Coast, 1995).

A BLAST search of the *Drosophila* genome database (Adams et al., 2000) revealed the presence of a gene product containing a sequence that is 71 % identical to Dippu-DH_{31}. The gene encoding this product is on chromosome 2L and maps cytologically to 29D1, for which there are no reported mutant alleles. The structure of the putative preprohormone is shown in Fig. 1. It is 116 amino acid residues long and commences with a 25-residue signal peptide. Appropriate N-terminal (KR) and C-terminal (GRR) processing sites for a 31-residue peptide amidated at the C terminus flank the Dippu-DH_{31}-like sequence. We describe the synthesis of this peptide, which we have called Drome-DH_{31} because of its homology with Dippu-DH_{31} (Fig. 1B). Drome-DH_{31} is shown to stimulate fluid secretion by *Drosophila melanogaster* tubules and activates an H^+-translocating vacuolar-type ATPase (V-ATPase) *via* cyclic AMP.

A

MTNRCACFAL AFLLFCLLAI SSIEAAPMPS QSNGGYGGAG
YNELEEVPDD LLMELMTRFG RTIIRARNDL ENS**KR**TVDFG
LARGYSGTQE AKHRMGLAAA NFAGGP**GRR**R RSETDV



B

Dippu-DH_{31} GL**DLGL**S**RG**F**SGSQ**A**AKHLMGLAAAN**Y-**AGGP-NH$_2$**
Drome-DH_{31} TV**DF**G**LA**R**G**Y**SGT**Q**EAKH**R**MGLAAAN**F-**AGGP-NH$_2$**
Chicken-CT CAS**L**STCVLGKL**SQE**LHK**L**QTYPRTDVG**AGT**P-NH$_2$**

Fig. 1. (A) The sequence of the preprohormone for the *Drosophila melanogaster* homologue (Drome-DH_{31}) of Dippu-DH_{31}. The 31 amino acid sequence of Drome-DH_{31} is underlined, and the two flanking sequences are shown in bold type. Below is a schematic representation of its organisation. The 25-residue signal peptide is cross-hatched. The amino acid sequence of Drome-DH_{31} (shaded) is flanked by appropriate residues (KR and GRR; in black) for the processing of the amidated peptide. (B) The aligned sequences of Dippu-DH_{31}, Drome-DH_{31} and chicken calcitonin (CT) performed using the program CLUSTAL W. Residues identical in any two peptides are highlighted.

Materials and methods

Insects

Adult male and female *Drosophila melanogaster* (wild-type stock collected in Dahomey, now Benin, West Africa, in 1970) were generously provided by Professor Linda Partridge from a culture maintained in The Galton Laboratory (Department of Biology, University College, London NW1 2HE). They were held at 25 °C on a 12 h:12 h light:dark cycle and were used 3–14 days post-emergence.

Peptide synthesis

Drome-DH_{31} was synthesised using *N*α-9-fluorenylmethoxycarbonyl (FMOC) chemistry with an Applied Biosystems 431A synthesiser (Foster City, CA, USA). The peptide was synthesized using Rink MBHA amide resin (Novabiochem, San Diego, CA, USA) on a 0.1 mmol scale, utilizing 1-hydroxybenzotriazole in 1-methyl-2-pyrrolidinone in the presence of dicyclohexylcarbodiimide for FMOC-amino acid activation. Extended 88 min coupling cycles with a 10-fold molar excess of acylating species were employed; no double coupling was performed. Protecting groups were Arg-[2,2,4,6,7-pentamethylbenzo-(2,3-dihydro)-furansulphonyl], Asn(Trityl), Asp(OtBu), Gln(Trityl), Glu(OtBu), Lys(Boc), Ser(OtBu), Thr(OtBu) and Tyr(OtBu). The dry resin–peptide was cleaved using reagent K (King et al., 1990). After precipitation with methyl-*t*-butyl ether, washing with methyl-*t*-butyl ether and drying, 334 mg of crude peptide was recovered.

Crude Drome-DH_{31} was purified using a Spectrasystem P4000 pump and Spectrasystem UV2000 detector set at 220 and 280 nm (Thermo Separation Products, San Jose, CA, USA) with a C_8 preparative column (20 mm×250 mm, 12 nm pores, YMC, Waters Associates, Milford, MA, USA) eluted at 15 ml min^{-1} with a linear gradient from 0.1 % trifluoroacetic acid (TFA) to 57 % ethanol/0.1 % TFA in water over a 60 min period. Certain fractions from this purification were homogeneous, whereas others were contaminated with either an Arg deletion peptide or a Thr deletion peptide. Fractions containing the Arg deletion peptide were purified to homogeneity using a PolyCAT A cation-exchange column (22 mm×250 mm, PolyLC Inc., Columbia, MD, USA) eluted with a multilinear gradient starting at 50 mmol l^{-1} $NH_4CH_3CO_2$, pH 5.5, 0 % acetic acid and ending at 30 % aqueous acetic acid. Fractions containing the Thr deletion peptide impurity were purified to homogeneity using a C_{18} column (20 mm×250 mm, 12 nm pores, YMC, Waters Associates, Milford, MA, USA) using a multilinear gradient starting with 0.1 % aqueous TFA and ending with 57 % ethanol/0.1 % TFA. The molecular mass of the synthetic peptide was determined by MALDI-MS (Bruker Proflex Plus, Billerica, MA, USA). Analyses were consistent with the synthetic peptide having the desired structure.

Fluid secretion assay

The diuretic assay was essentially the same as that described previously (Dow et al., 1994) except that both anterior- and posterior-directed tubules were used and the bathing fluid volume was reduced to 5 µl. The dissection saline had the following composition (in mmol l^{-1}): 135 NaCl, 20 KCl, 2 $CaCl_2$, 8.5 $MgCl_2$, 10.2 $NaHCO_3$, 4.3 NaH_2PO_4, 15 Hepes, 20 glucose, pH 7.0, and was diluted 1:1 with Schneider's *Drosophila* medium to prepare the standard bathing fluid for the diuretic assay. For some experiments, the Cl^- concentration of the bathing fluid was reduced. For these studies, tubules were bathed in dissection saline diluted as appropriate with Cl^--free saline (gluconate replacing Cl^-).

Tubules were allowed to equilibrate for 30–60 min before the bathing fluid was replaced and droplets of secreted urine removed. Urine was then collected over 10–45 min intervals before and after the addition of diuretics. Each tubule therefore served as its own control, and diuretic activity could be determined as the change in the rate of secretion (Δnl min^{-1}). All experiments were performed at room temperature, which varied between 22 and 26 °C.

Measurement of cyclic AMP and cyclic GMP production

Malpighian tubules were transferred to Eppendorf tubes (two pairs per tube) containing 44 µl of *Drosophila* saline. After a 10 min incubation at room temperature, 5 µl of saline containing 5 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX; final concentration 0.5 mmol l^{-1}) was added to each tube followed 10 min later by 1 µl of saline containing Drome-DH_{31}. The IBMX solution was freshly prepared from a 250 mmol l^{-1} stock solution in dimethylsulphoxide (DMSO; final concentration 0.2 %). The incubation was terminated after 10 min by the addition of 50 µl of ice-cold sodium acetate buffer (pH 4.8) containing EDTA and IBMX (final concentrations: 50 mmol l^{-1} sodium acetate; 20 mmol l^{-1} EDTA; 0.5 mmol l^{-1} IBMX). The tubes were immediately quenched in liquid nitrogen and stored at –80 °C. Samples were sent on dry ice to the University of North Wales within 4 days of the experiment for the measurement of cyclic AMP and cyclic GMP.

After thawing, the samples were sonicated (Soniprep 150, MSE Scientific Instruments, Crawley, UK) for 10 s on ice to ensure release of intracellular cyclic nucleotides. Samples containing 0.4 tubule equivalents (cyclic AMP) and 0.8 tubule equivalents (cyclic GMP) were acetylated prior to radioimmunoassay (Brooker et al., 1979). ^{125}I-labelled probes (specific activity 27–37 TBq mmol^{-1}) were prepared by chloramine-T iodination of the corresponding 2′-2-*O*-succinyl methyl esters (following the method of Bolton, 1989). The labelled products were separated from unincorporated ^{125}I by elution on a Sep-Pak C_{18} cartridge (Waters, Milford, USA) equilibrated with water and eluted in 40 % isopropanol. Rabbit anti-cyclic-AMP antiserum (final dilution 1:4000) and anti-cyclic-GMP antiserum (final dilution 1:24 000) were generously provided Dr J. de Vente (Maastricht University, The Netherlands). The separation of bound from free ligand was performed using solid-phase donkey anti-rabbit IgG (Sac-Cel, Immunodiagnostic Services, Tyne and Wear, UK).

Electrophysiological studies

Techniques for recording transepithelial (V_{tep}) and basolateral membrane (V_{bl}) potentials were essentially as described previously (O'Donnell et al., 1996). Microelectrodes (10–20 MΩ resistance when filled with electrolyte) were backfilled with 3 mol l^{-1} KCl and connected to a high-impedance electrometer (M-707A, World Precision Instruments) *via* a Ag/AgCl half-cell (World Precision Instruments). Dissected tubules were transferred to 50 µl drops of standard bathing fluid in a Petri dish lined with Sylgard, to which they readily adhered. Recordings were made from the distal end of the main (secretory) segment, it having been shown (O'Donnell et al., 1996) that V_{bl} and V_{tep} are relatively constant within this segment. Principal cells were impaled by advancing a microelectrode at an oblique angle using a hydraulic micromanipulator (MMO-203, Narishigi, Tokyo, Japan) until a sudden jump in potential indicated that the basolateral membrane had been penetrated. Recordings were deemed successful if the potential remained stable (±2 mV) for more than 30 s and returned to 0±2 mV after withdrawal of the electrode. Similar criteria were adopted for recording V_{tep} after the microelectrode had been advanced through the apical membrane into the tubule lumen.

Measurements of urine K^+ concentration and pH

The K^+ concentration and pH of secreted urine were measured using ion-selective microelectrodes (as described by O'Donnell et al., 1996). Ion-selective electrodes were silanized by exposure to dimethyldichlorosilane vapour for 10–20 min at 200 °C (Wright and O'Donnell, 1992). K^+ electrodes were based on Corning 477317 liquid ion-exchange resin and were back-filled with 1.5 mol l^{-1} KCl. Electrodes used for the measurement of pH were based on a hydrogen ion exchange resin (IE 010, World Precision Instruments) and were back-filled with 0.5 mol l^{-1} citric acid containing 10 mmol l^{-1} NaCl adjusted to pH 6.0.

Urine droplets were transferred to a Sylgard-lined Petri dish containing water-saturated liquid paraffin together with 1 µl drops of the calibration solutions. For K^+-selective electrodes, the following calibration solutions were used (in mmol l^{-1}): 148 KCl:2 NaCl, 120 KCl:30 NaCl and 80 KCl: 70 NaCl. The calibration solutions for pH microelectrodes were prepared by titrating standard reference buffers (pH 7.0 and 9.0; Sigma) with 1 mol l^{-1} NaOH or HCl to a pH of 7.14, 7.62 or 8.15. Reference and ion-selective electrodes were positioned in the same droplet, and potentials were recorded using a high-impedance electrometer (F-223A, World Precision Instruments). Electrodes were deemed acceptable if the calibration curve was linear with a slope per decade change in ion concentration of at least 52 mV (K^+) or at least 50 mV (H^+). Results are given as concentrations rather than ion activities, following a similar convention to that used in other studies (O'Donnell et al., 1996).

Chemicals

Muscakinin (Musdo-K, which differs from Drome-K by the conservative substitution of threonine for serine at residue two) was a generous gift from Dr R. J. Nachman (U.S.D.A. College Station, TX, USA). Unless stated otherwise, all other chemicals were obtained from Sigma-Aldrich (Poole, Dorset).

Statistical analyses

Results are expressed as mean values ± S.E.M. for (*N*) determinations. Tests for significance were performed using GraphPad Instat 3.01 (GraphPad Software, San Diego, CA, USA), with $P<0.05$ being accepted as significant. Dose–response curves with variable slope were fitted using Prism (GraphPad Software).

Results

Drome-DH$_{31}$ stimulates tubule secretion

In the present study, the basal rate of secretion by *Drosophila melanogaster* tubules was 0.38±0.01 nl min^{-1} (N=317; range 0.07–0.91 nl min^{-1}). This is lower than the value of 0.56±0.01 nl min^{-1} reported previously (Riegel et al., 1999), but these authors note the extreme variability, with fluid secretion values ranging from 0.11 to 2.06 nl min^{-1} in their study. Addition of 50 nmol l^{-1} Drome-DH$_{31}$ doubled the rate of secretion (Fig. 2A); the stimulated rate was doubled again when 20 nmol l^{-1} Musdo-K was included in the bathing fluid. Fig. 2B shows results from a similar experiment in which 20 nmol l^{-1} Musdo-K was added prior to Drome-DH$_{31}$. Musdo-K produced a fourfold increase in secretion, which was increased further by the addition of 50 nmol l^{-1} Drome-DH$_{31}$, although the latter increase was not significant. Dose–response curves for Drome-DH$_{31}$ and Musdo-K are presented in Fig. 3. Drome-DH$_{31}$ increased secretion to a maximum of 0.75±0.01 nl min^{-1}, which is less than 35 % of that produced by Musdo-K (2.15±0.04 nl min^{-1}); their EC_{50} values are 4.3 nmol l^{-1} for Drome-DH$_{31}$ (95 % confidence interval 3.0–6.1 nmol l^{-1}) and 1.4 nmol l^{-1} for Musdo-K (95 % confidence interval 1.1–1.6 nmol l^{-1}). The EC_{50} for Musdo-K is approximately an order of magnitude greater than that reported for Drome-K (Terhzaz et al., 1999). The dose–response curve for Musdo-K is significantly ($P<0.01$) steeper (Hill slope 1.96±0.25) than that for Drome-DH$_{31}$ (Hill slope 0.71±0.07), suggesting a difference in the relationship between receptor binding and cellular response for the two peptides.

Dippu-DH$_{31}$ and chicken calcitonin were also tested on *Drosophila melanogaster* tubules at a single dose of 1 µmol l^{-1}, which was sufficient to more than double the rate of secretion by *Diploptera punctata* tubules (Furuya et al., 2000). Calcitonin had no effect on fluid secretion (Δ –0.03±0.02 nl min^{-1}; N=5; P=0.225, paired t-test), although the tubules responded to the subsequent addition of 50 nmol l^{-1} Drome-DH$_{31}$ (Δ 0.20±0.02 nl min^{-1}; N=5; $P<0.005$). However, Dippu-DH$_{31}$ (1 µmol l^{-1}) significantly increased fluid secretion (Δ 0.13±0.02 nl min^{-1}; N=9; $P<0.001$) and, although Drome-DH$_{31}$ (50 nmol l^{-1}) produced a further increase (Δ 0.04±0.04 nl min^{-1}; N=9), this was not significant (P=0.347).

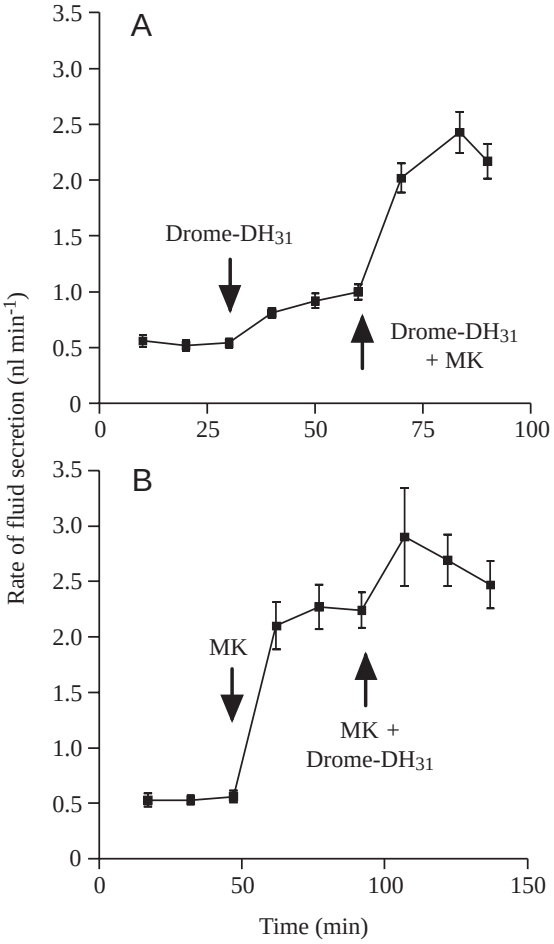


Fig. 2. The effects of (A) 50 nmol l^{-1} Drome-DH$_{31}$ and (B) 20 nmol l^{-1} Musdo-K (MK) tested separately and together on isolated Malpighian tubules. The diuretic response to Drome-DH$_{31}$ (A; first arrow) is considerably less than that obtained with Musdo-K (B; first arrow). In each experiment, tubule secretion is increased further when the two peptides are applied together (A,B; second arrow). Data points are the means and vertical lines indicate ±1 S.E.M. for (A) 13 and (B) six determinations.

Additive effects of Drome-DH$_{31}$ and muscakinin

To determine whether the activities of Drome-DH$_{31}$ and Musdo-K were additive or synergistic, they were each tested separately and then together at threshold concentrations of 5 nmol l^{-1} for Drome-DH$_{31}$ and 0.1 nmol l^{-1} for Musdo-K. Drome-DH$_{31}$ increased tubule secretion by 0.14±0.04 nl min^{-1} (N=6; $P<0.05$, paired t-test) and Musdo-K by 0.21±0.06 nl min^{-1} (N=6; $P<0.05$, paired t-test) compared with the period immediately prior to peptide addition. When the two peptides were added together, there was a further increase in the rate of secretion, but the combined response (Δ 0.44±0.06 nl min^{-1}; N=14) was not significantly greater than the sum of their separate activities and therefore provides no evidence for synergism. In a separate experiment, tubules were

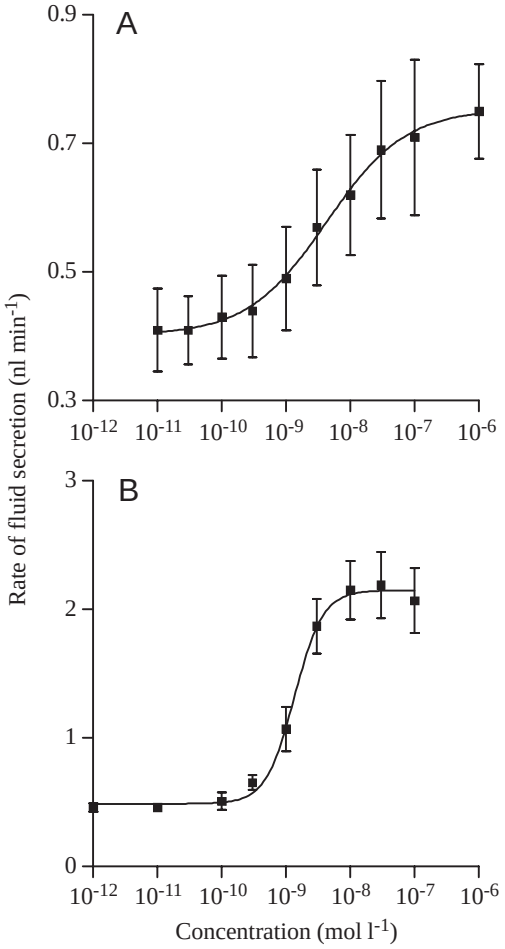


Fig. 3. Dose–response curves for the diuretic activities of (A) Drome-DH_{31} and (B) Musdo-K. The maximal response to Drome-DH_{31} is less than 35 % of that to Musdo-K, and the EC_{50} values are 4.3 nmol l^{-1} for Drome-DH_{31} and 1.4 nmol l^{-1} for Musdo-K. Data points are the means and vertical lines indicate ±1 S.E.M. for 6–11 determinations.

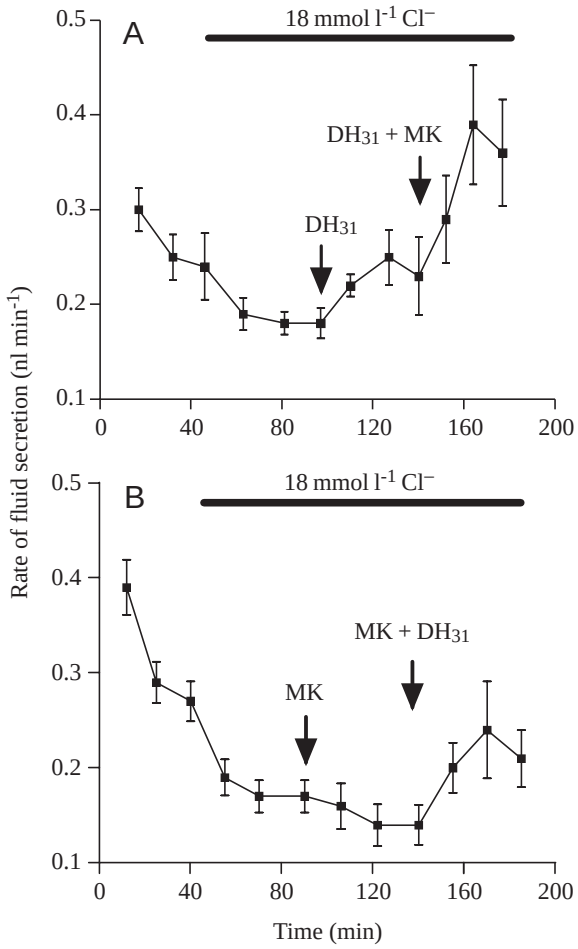


Fig. 4. The effects of low-Cl^- saline on the separate and combined activities of Drome-DH_{31} (DH_{31}) and Musdo-K (MK). In A, Drome-DH_{31} was added prior to Musdo-K, whereas this order was reversed in B. Secretion slows after tubules are transferred to dissection saline, and the rate drops still further when they are moved to saline with a reduced Cl^- concentration (18 mmol l^{-1}; solid bar). The addition of 50 nmol l^{-1} Drome-DH_{31} (A; first arrow) increases tubule secretion, and it is increased still further when 20 nmol l^{-1} Musdo-K is also added (A; second arrow). In contrast, 20 nmol l^{-1} Musdo-K alone has no effect on Malpighian tubules bathed in low-Cl^- saline (B; first arrow), but fluid secretion is stimulated when Drome-DH_{31} is also added to the bathing fluid (B; second arrow). Data points are the means and vertical lines indicate ±1 S.E.M. for 6–10 determinations.

first challenged with a supra-maximal concentration (100 nmol l^{-1}) of Musdo-K and then together with 50 nmol l^{-1} Drome-DH_{31}. Tubule secretion was increased from 0.40±0.08 to 1.88±0.30 nl min^{-1} (N=5) by Musdo-K and reached 2.36±0.38 nl min^{-1} after the addition of Drome-DH_{31}. The mean difference in the rate of secretion before and after the addition of Drome-DH_{31} was 0.48±0.15 nl min^{-1} (P<0.05, paired t-test).

The fact that Drome-DH_{31} can increase the rate of secretion by tubules that are already maximally stimulated with Musdo-K indicates a difference in their mode of action, even though we could find no evidence for synergism. Kinins are known to act by opening a cellular or paracellular Cl^- conductance (Pannabecker et al., 1993; Wang et al., 1996; O'Donnell et al., 1998), and the resultant increase in fluid secretion is dependent on a favourable electrochemical gradient for the passive entry of Cl^- into the tubule lumen. In an attempt to distinguish between the activities of Drome-DH_{31} and Musdo-K, they were tested for diuretic activity in low-Cl^- saline prepared by a 10-fold dilution of dissection saline (which has a Cl^- concentration of 176 mmol l^{-1}) with Cl^--free saline. Tubule secretion fell to 0.28±0.02 nl min^{-1} (N=17) in dissection saline, probably because it contains no amino acids, which have been implicated in solute-coupled Na^+ entry across the basolateral membrane (Linton and O'Donnell, 1999). Transfer to low-Cl^- saline resulted in a further decrease in the rate of secretion (Fig. 4A,B), reflecting the reduced electrochemical gradient for Cl^- diffusion into the tubule lumen. However, fluid secretion was stimulated by the addition of 50 nmol l^{-1} Drome-DH_{31} (Δ 0.04±0.01 nl min^{-1}; N=8; paired t-test, t=2.857) and was

further increased by the inclusion of 20 nmol l^{-1} Musdo-K in the bathing fluid (Fig. 4A). In contrast, Musdo-K alone had no effect on the rate of urine production by tubules bathed in low-Cl^- saline, although fluid secretion was increased by 0.07±0.01 nl min^{-1} (paired *t*-test, *t*=5.00, d.f.=6) in the first collection period after the addition of Drome-DH_{31} (Fig. 4B).

Effects of Drome-DH_{31} on transepithelial and basolateral membrane potentials

The mean basolateral membrane potential (V_{bl}) recorded from principal cells was –41.9±0.8 mV from 44 impalements of different tubules, while the mean transepithelial potential (V_{tep}) across the main secretory segment was +36.8±2.5 mV (*N*=23 impalements of different tubules). Hence, the apical membrane potential ($V_{ap}=V_{tep}-V_{bl}$) is approximately +80 mV. These values are somewhat lower than those recorded previously (O'Donnell et al., 1996) from Malpighian tubules of Oregon R strain female flies. Drome-DH_{31} (final concentration 50 nmol l^{-1}) had no significant effect on V_{bl} (Fig. 5A), which depolarised by 0.6±0.3 mV (*N*=12; Fig. 6A). In contrast, V_{bl} immediately depolarised when the K^+ concentration of the bathing fluid was increased approximately fourfold by the addition of 3 mol l^{-1} KCl (Fig. 5A), indicative of a basolateral membrane K^+ conductance, as described previously in *Drosophila melanogaster* tubules (O'Donnell et al., 1996). A sample recording demonstrating the effect of Drome-DH_{31} on V_{tep} is shown in Fig. 5B. The transepithelial potential increased from +56 mV to +67 mV after the addition of Drome-DH_{31} (final concentration 50 nmol l^{-1}). The mean change in V_{tep} within 2 min of adding the peptide was +9.4±1.1 mV (*N*=13 impalements of different tubules; Fig. 6A), which is highly significant (*P*<0.0001) in a paired *t*-test. The subsequent addition of Musdo-K (final concentration 10 nmol l^{-1}) resulted in an immediate depolarisation of V_{tep}, as reported previously for the effect of leucokinins in *Drosophila melanogaster* and *Aedes aegypti* tubules (O'Donnell et al., 1996; Pannabecker et al., 1993). The potential profile across a principal cell before and after stimulation with 50 nmol l^{-1} Drome-DH_{31} is shown in Fig. 6B. The apical membrane potential is increased from +79 mV to +86 mV (lumen positive) by Drome-DH_{31} while V_{bl} is essentially unchanged. The hyperpolarisation of V_{tep} by Drome-DH_{31} is similar to that

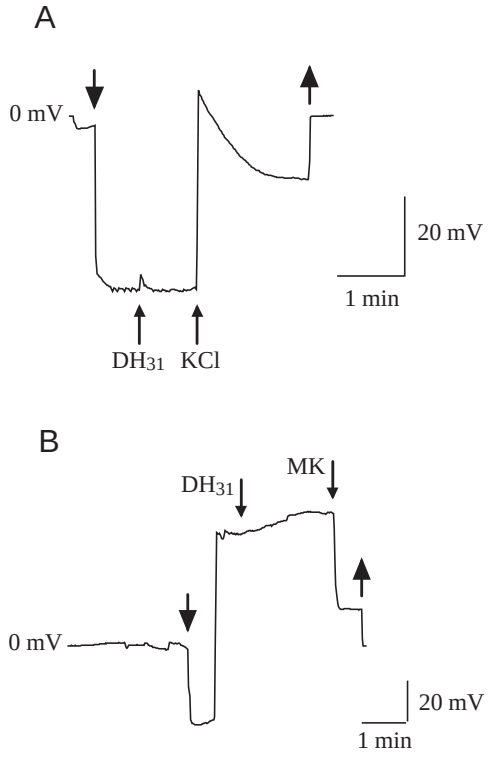


Fig. 5. (A) Sample recordings showing the effect of 50 nmol l^{-1} Drome-DH_{31} (DH_{31}) on principal cell basolateral membrane potential and (B) on the transepithelial potential across the main secretory tubule segment. The large downward arrows indicate when the microelectrode was advanced into the cell in A and into the tubule lumen in B, while large upward arrows indicate electrode withdrawal into the bathing fluid. Smaller arrows show the time of addition of Drome-DH_{31} (final concentration 50 nmol l^{-1}) and 2 µl of 3 mol l^{-1} KCl (A) or Musdo-K (MK) (B; final concentration 10 nmol l^{-1}).

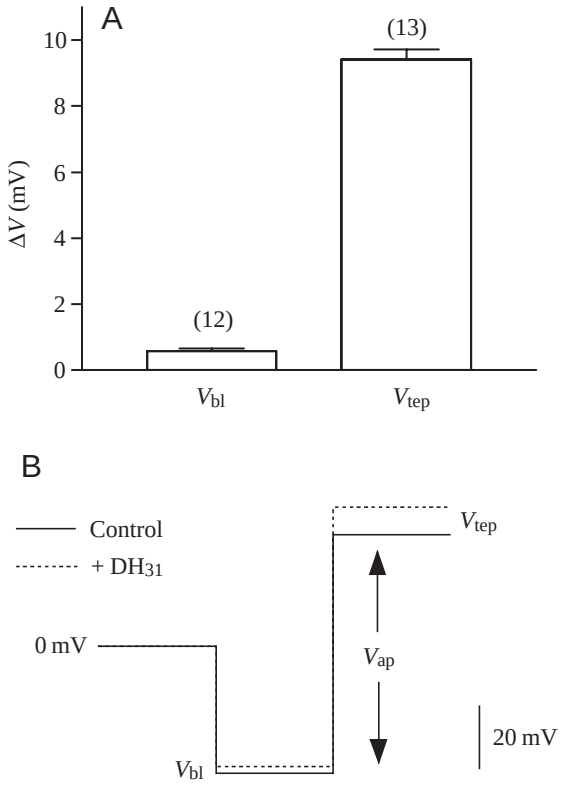


Fig. 6. (A) A summary of the effects of 50 nmol l^{-1} Drome-DH_{31} on principal cell basolateral membrane potential (V_{bl}) and on the transepithelial potential (V_{tep}) across the main secretory tubule segment. Columns represent the means and vertical lines +1 S.E.M. for the number of determinations shown in parentheses. (B) The potential profile across a principal cell in the main secretory segment before (solid line) and after (dotted line) the addition of 50 nmol l^{-1} Drome-DH_{31} (DH_{31}). V_{ap}, apical membrane potential.

reported previously (O'Donnell et al., 1996) following the addition of cyclic AMP, cyclic GMP or CAP_{2b}, suggesting that all three secretagogues are targeted to the same transport process, probably the apical V-ATPase. Indeed, Drome-DH_{31} had no effect on V_{tep} in tubules previously stimulated with either 0.5 mmol l^{-1} cyclic GMP or 1 mmol l^{-1} cyclic AMP (results not shown).

Consistent with the observations of O'Donnell et al. (O'Donnell et al., 1996), V_{bl} was slightly lower for tubules bathed in low-Cl^- saline (–37.9±1.0 mV from 16 impalements of different tubules), whereas V_{tep} was higher (+47.5±2.9 mV; N=8). Drome-DH_{31} (final concentration 50 nmol l^{-1}) had no effect on V_{bl} (Δ +0.5±0.6 mV; N=4), but V_{tep} increased by +14.3±2.0 mV (N=8), which was significantly (P<0.05) greater than in the standard bathing medium (see above). The apical membrane potential is therefore increased from +85 mV to +100 mV by Drome-DH_{31}. When stimulated with Drome-DH_{31}, the increase in V_{tep} establishes a favourable electrochemical potential gradient for Cl^- to enter the lumen from the low-Cl^- bathing fluid, even if it is the only anion present in the secreted urine. Prior to the addition of Drome-DH_{31}, the gradient is small and in the opposite direction.

Effects of cyclic AMP and cyclic GMP on the response to Drome-DH_{31}

If Drome-DH_{31}, cyclic AMP and cyclic GMP do target the same transport process, Drome-DH_{31} should have no effect on fluid secretion in tubules already stimulated maximally with cyclic AMP or cyclic GMP. To test this, tubules were challenged with either 1 mmol l^{-1} 8-bromo-cyclic AMP or 0.5 mol l^{-1} cyclic GMP alone and then in combination with 50 nmol l^{-1} Drome-DH_{31}. The rate of urine production was significantly increased by both of the cyclic nucleotides, but was not increased further by the inclusion of Drome-DH_{31} in the bathing fluid (Fig. 7).

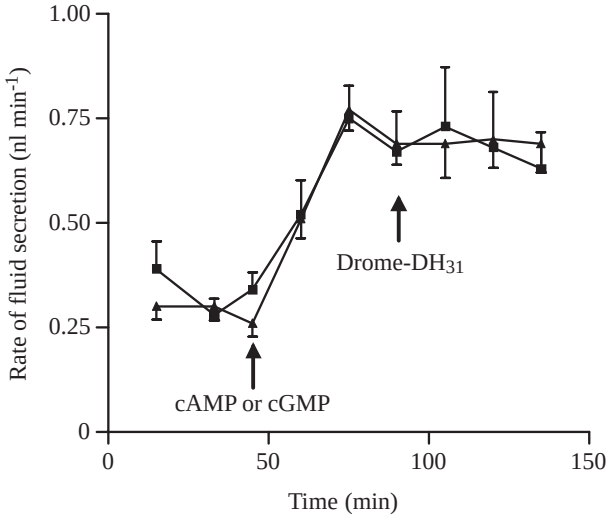


Fig. 7. The effects of 50 nmol l^{-1} Drome-DH_{31} on fluid secretion by Malpighian tubules previously stimulated with either 1 mmol l^{-1} cyclic AMP (filled squares) or 0.5 mmol l^{-1} cyclic GMP (filled triangles). The first arrow shows the time of addition of the cyclic nucleotide and the second arrow when Drome-DH_{31} was also added. Data points are the means and vertical lines indicate ±1 S.E.M. of 4–6 determinations.

Effects of Drome-DH_{31} on cyclic AMP and cyclic GMP production

To determine whether Drome-DH_{31} acts *via* cyclic AMP or cyclic GMP, cyclic nucleotide production was measured in the presence of 0.5 mmol l^{-1} IBMX, a non-specific phosphodiesterase inhibitor. After a 10 min incubation in IBMX saline, Drome-DH_{31} (final concentration 50 nmol l^{-1}) or saline alone (controls) was added to the medium, and the incubation was allowed to proceed for a further 10 min before it was terminated by the addition of ice-cold sodium acetate buffer. Intracellular cyclic nucleotides were released by sonication and measured in a radioimmunoassay. The results are presented in Fig. 8. The amount of cyclic AMP present in control tubules was 175±9 fmol tubule^{-1} (N=16) compared

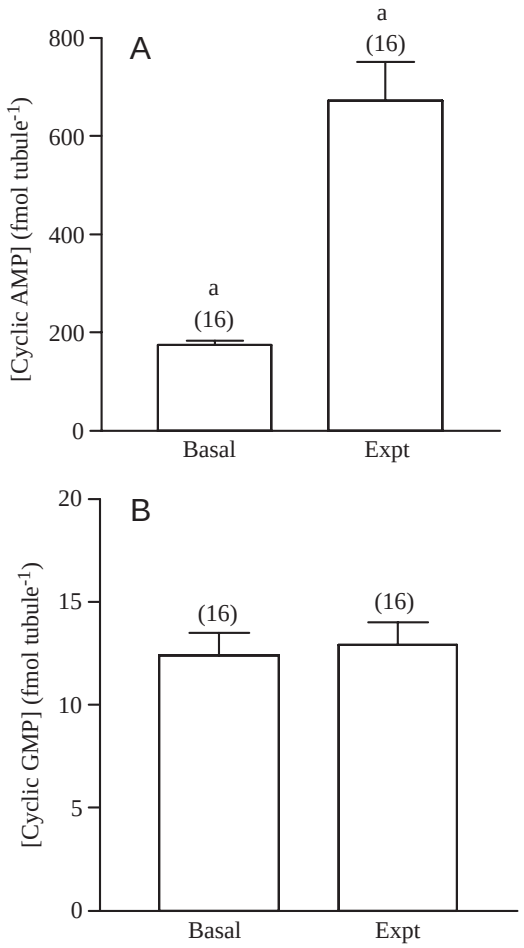


Fig. 8. The effect of 50 nmol l^{-1} Drome-DH_{31} on the production of cyclic AMP (A) and cyclic GMP (B) in the presence of 0.5 mmol l^{-1} IBMX. Columns represent the means and vertical lines indicate +1 S.E.M. for 16 determinations in the absence (Basal) and presence (Expt) of Drome-DH_{31}. Identical letters indicate values that are significantly different.

with 12.4±1.1 fmol tubule^{-1} (N=16) of cyclic GMP. These values are approximately an order of magnitude greater than those reported by Davies et al. (Davies et al., 1995), who incubated tubules in Schneider's *Drosophila* medium containing only 0.1 mmol l^{-1} IBMX. In addition, Davies et al. (Davies et al., 1995) extracted cyclic nucleotides in ethanol before drying and redissolving in sodium acetate buffer, which may be considerably less efficient than direct extraction in buffer. Regardless of the absolute levels of cyclic nucleotides present, stimulation with Drome-DH$_{31}$ resulted in a fourfold increase in cyclic AMP production, whereas cyclic GMP production was unchanged (Fig. 8).

In a separate experiment, Drome-DH$_{31}$ was tested at concentrations of between 0.01 and 100 nmol l^{-1} for its effect on cyclic AMP production following the protocol outlined above. The results are presented in Fig. 9 and show a dose-dependent stimulation of cyclic AMP production; the EC$_{50}$ is 0.7 nmol l^{-1} (95 % confidence interval 0.1–3.9 nmol l^{-1}) compared with 4.3 nmol l^{-1} (95 % confidence interval 3.0–6.1 nmol l^{-1}) in the fluid secretion assay (see above). The small difference in the potency of Drome-DH$_{31}$ in the two bioassays is probably due to differences in the experimental conditions, most notably the inclusion of IBMX in the cyclic AMP assay. For example, when IBMX was included in a fluid secretion assay, the potency of a CRF-related diuretic peptide that acts *via* cyclic AMP was increased (Coast and Kay, 1994).

Effects of Drome-DH$_{31}$ on urine pH and K$^+$ concentration

O'Donnell et al. (O'Donnell et al., 1996) propose that cyclic AMP and cyclic GMP stimulate fluid secretion by activating an electrogenic proton-translocating V-ATPase in the apical membrane of principal cells. To investigate whether Drome-DH$_{31}$ increased apical V-ATPase activity, we determined its effect on the pH and K$^+$ concentration of secreted urine. The pH fell from 7.68±0.08 (N=12) to 7.36±0.05 (N=14) after the addition of 50 nmol l^{-1} Drome-DH$_{31}$ (bathing fluid pH 7.08±0.08; N=15). When samples were collected from the same tubule before and after stimulation by Drome-DH$_{31}$, the mean change in urine pH was –0.25±0.05 (N=10), which is highly significant (paired t-test; t=5.00). The effect of Drome-DH$_{31}$ is very similar to that reported previously (O'Donnell et al., 1996) for tubules stimulated with 1 mmol l^{-1} cyclic AMP; in that study, the pH of urine from the main tubule segment was 7.74±0.07 before and 7.38±0.07 after addition of the cyclic nucleotide.

In a separate experiment, the K$^+$ concentration of urine samples was measured before and after stimulation with 50 nmol l^{-1} Drome-DH$_{31}$. The K$^+$ concentration of urine secreted by unstimulated tubules was 148.3±6.1 mmol l^{-1} (N=14) compared with 150.5±4.7 mmol l^{-1} (N=13) after the addition of Drome-DH$_{31}$. The K$^+$ concentration of the standard bathing fluid was 21.9±0.5 mmol l^{-1} (N=14). Where urine samples had been collected from the same tubule before and after the addition of Drome-DH$_{31}$, the K$^+$ concentration increasd by 1.1±2.5 mmol l^{-1} (N=12), which is not significant. Although Drome-DH$_{31}$ had no effect on the K$^+$ concentration of secreted urine, it increased the rate of K$^+$ transport (the product of concentration and urine flow) from 59.5±7.6 pmol min^{-1} (N=14) to 104.6±4.6 pmol min^{-1} (N=13), which is highly significant (P<0.0001). O'Donnell et al. (O'Donnell et al., 1996) obtained values of 128.3±5.1 mmol l^{-1} and 125.7±4.8 mmol l^{-1} for the K$^+$ concentration of urine samples collected from the main tubule segment before and after stimulation with 1 mmol l^{-1} cyclic AMP. Although these values are lower than those reported in the present study, the results are qualitatively identical in that both Drome-DH$_{31}$ and cyclic AMP increase K$^+$ transport without affecting the urine K$^+$ concentration.

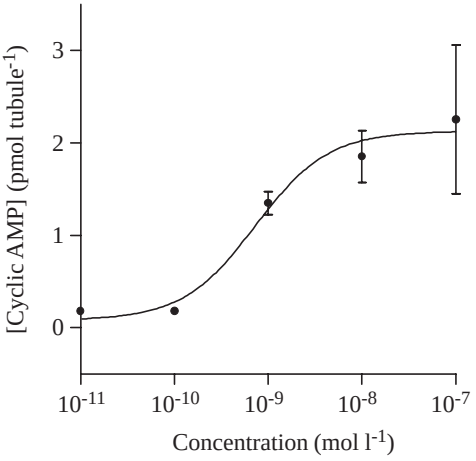


Fig. 9. The dose-dependent stimulation of cyclic AMP production by Drome-DH$_{31}$ in the presence of 0.5 mmol l^{-1} IBMX. Data points are the means and vertical lines indicate ±1 S.E.M. for six determinations. Low concentrations (0.01 and 0.1 nmol l^{-1}) of Drome-DH$_{31}$ have no effect on cyclic AMP production, while higher concentrations produce a seven- to 12-fold increase. The EC$_{50}$ is 0.7 nmol l^{-1}.

Discussion

The gene encoding Drome-DH$_{31}$ was identified in a BLAST search of the *Drosophila* genome database (Adams et al., 2000) using as a query the sequence of Dippu-DH$_{31}$, a calcitonin-like diuretic peptide from *Diploptera punctata* (Furuya et al., 2000). The gene product encodes a preprohormone that begins with a signal peptide and contains a 31-residue sequence that is 71 % identical with Dippu-DH$_{31}$ (87 % allowing for conservative substitutions) flanked by appropriate residues for processing the amidated peptide. The similarity between calcitonin and Drome-DH$_{31}$ (17 % identical) is lower than for Dippu-DH$_{31}$ (23 % identical), but the *Drosophila melanogaster* preprohormone has a similar organisation to the preprohormone of calcitonin and they share 16–20 % sequence identity. Such low sequence identity might result from chance rather than from shared ancestry. However, it is noteworthy that chicken calcitonin has diuretic activity on *Diploptera punctata* tubules, although it is not active in *Locusta migratoria* (Furuya et al., 2000) or *Drosophila melanogaster* (this study).

We have synthesised Drome-DH_{31} and investigated its diuretic activity in Malpighian tubules from *Drosophila melanogaster*. The maximum response to Drome-DH_{31} is approximately 35 % of that produced by Musdo-K, a highly potent stimulant of *Drosophila melanogaster* tubule secretion that differs from Drome-K by a conservative substitution of threonine for serine at residue 2 (Holman et al., 1999; Terhzaz et al., 1999). Similarly, Dippu-DH_{31} gave less than 50 % maximal stimulation of *Diploptera punctata* and *Locusta migratoria* tubules (Furuya et al., 2000), although a maximal response is here defined by the diuretic activity of a CRF-related peptide (Dippu-DH_{46} and Locmi-DH, respectively), whereas kinins are the most effective stimulants of tubule secretion in *Drosophila melanogaster*. Although Drome-DH_{31} and Dippu-DH_{31} have only a modest effect on tubule secretion, their potency (EC_{50} ranging from 0.6 to 9.8 nmol l^{-1}) is typical of a neurohormone.

The activities of Drome-DH_{31} and Musdo-K are additive at supra-maximal concentrations, which suggests they stimulate different transport processes. In support of this, Drome-DH_{31} stimulates secretion by tubules bathed in low-Cl^- saline, whereas Musdo-K has no effect unless added with the calcitonin-like peptide. Kinins act by opening a cellular or paracellular conductance pathway (Pannabecker et al., 1993; Wang et al., 1996; O'Donnell et al., 1998), and any reduction in the electrochemical gradient favouring Cl^- movement into the lumen is therefore expected to blunt the response to Musdo-K. However, this will have little effect on the response to Drome-DH_{31}, which stimulates active cation transport (see below).

Several lines of evidence suggest that Drome-DH_{31} stimulates cation transport across principal cells in the main secretory segment of Malpighian tubules by activating an apical membrane V-ATPase *via* an increase in intracellular cyclic AMP levels. First, Drome-DH_{31} stimulates cyclic AMP production by isolated Malpighian tubules in a dose-dependent manner, but has no effect on cyclic GMP production. Second, the diuretic activities of exogenous 8-bromo-cyclic AMP and Drome-DH_{31} are identical and are not additive, which suggests that they target the same transport process. Third, both Drome-DH_{31} and exogenous cyclic AMP (O'Donnell et al., 1996), together with cyclic GMP (Davies et al., 1995), hyperpolarise the apical membrane potential (V_{ap}) of principal cells in the main secretory segment. This can be attributed to activation of an apical V-ATPase, because stimulation of an apical proton/cation antiporter with a stoichiometry of 1 cation for $n H^+$ (where n is ≥1; Wieczorek et al., 1991; Leyssens et al., 1993) would either be electroneutral or would depolarise V_{ap}. Furthermore, after stimulation with Drome-DH_{31} or exogenous cyclic AMP (O'Donnell et al., 1996), the secreted urine is more acid, which would be expected from activation of the V-ATPase, whereas increased antiporter activity would have an alkalizing effect. In marked contrast, exogenous cyclic AMP analogues have no effect on the pH of urine secreted by *Aedes aegypti* tubules, but acidify the cytoplasm of principal cells, which has been attributed to activation of a $Na^+/2H^+$ antiporter in the apical membrane (Petzel et al., 1999).

Drome-DH_{31} doubles the rate of K^+ (plus Cl^-) transport into the tubule lumen; with a corresponding increase in fluid movement, the urine K^+ concentration is left unchanged. The elegant studies of O'Donnell et al. (O'Donnell et al., 1996) and Linton and O'Donnell (Linton and O'Donnell, 1999) have led to a model for the control of *Drosophila melanogaster* urine secretion by a ligand(s) acting *via* cyclic AMP. The V-ATPase of principal cells provides the driving force for K^+ and Cl^- transport into the lumen *via* cation/proton antiports and Cl^- channels in the apical membrane. This is coupled to KCl entry across the basolateral membrane *via* a K^+/Cl^- cotransporter driven by a favourable electrochemical gradient for Cl^- (Linton and O'Donnell, 1999). When the V-ATPase is stimulated by a rise in the intracellular level of cyclic AMP, the apical membrane potential is increased. Assuming that Cl^- is passively distributed across this surface, the intracellular concentration will fall (as predicted by the Nernst equation), which will increase the gradient for Cl^- and K^+ cotransport across the basolateral membrane (Linton and O'Donnell, 1999). The effect of Drome-DH_{31} on the transepithelial potential (and hence on V_{ap}) of tubules bathed in low-Cl^- saline is greater than in the standard bathing medium, which is consistent with the presence of a Cl^- conductance in the principal cell apical membrane. Moreover, the increase in V_{ap} is sufficient to maintain a favourable gradient for Cl^- and K^+ cotransport across the basolateral membrane, even from low-Cl^- saline. This not only accounts for the diuretic activity of Drome-DH_{31} in low-Cl^- saline, but also explains why tubules can only then respond to Musdo-K (see above), because the increased transepithelial potential favours the passive diffusion of Cl^- into the lumen through a shunt conductance situated outside the principal cells.

At present, there is no evidence to suggest that Drome-DH_{31} has any effect on the apical Cl^- conductance of principal cells or on the K^+/Cl^- cotransporter in the basolateral membrane. Linton and O'Donnell (Linton and O'Donnell, 1999) found that ouabain had a small inhibitory effect on fluid secretion by *Drosophila melanogaster* tubules challenged with exogenous cyclic AMP, which suggests that the cyclic nucleotide directly stimulates Na^+/K^+-ATPase activity in the basolateral membrane. We did not examine the effect of ouabain on the response to Drome-DH_{31}, but stimulation of the Na^+/K^+-ATPase would be expected to hyperpolarise V_{bl} and to increase the K^+ concentration of the secreted urine, neither of which was observed. The actions of Drome-DH_{31} in fruit fly tubules appear to be mediated solely *via* cyclic AMP whereas, in *Locusta migratoria*, Dippu-DH_{31} has kinin-like activity and acts synergistically with both Locmi-DH and exogenous 8-bromo-cyclic AMP (Furuya et al., 2000), which suggests that it uses an alternative second-messenger pathway.

In summary, the *Drosophila melanogaster* homologue of the cockroach calcitonin-like diuretic peptide Dippu-DH_{31} stimulates Malpighian tubule fluid secretion by activating the apical membrane V-ATPase of principal cells in the main secretory segment. Drome-DH_{31} is the second diuretic peptide identified in the fruit fly, but the first to be shown to

act *via* cyclic AMP. A third peptide that stimulates secretion by *Drosophila melanogaster* tubules is a cardioacceleratory peptide (Manse-CAP_{2b}) from *Manduca sexta*. It too increases apical V-ATPase activity, but does so *via* a cyclic-GMP-dependent mechanism (Davies et al., 1995; O'Donnell et al., 1996; Rosay et al., 1997). BLAST searches of the *Drosophila* genome database reveal putative peptides resembling Manse-CAP_{2b} and the insect CRF-related diuretic peptide family. The latter are known to act *via* cyclic AMP (Coast and Kay, 1994), so the fruit fly may have three different neuropeptides all acting to stimulate the apical membrane V-ATPase of principal cells. At present, we can offer no explanation for why this should be necessary, and it has still to be determined whether any of these peptides functions as a diuretic hormone in *Drosophila melanogaster*. However, since calcitonin-like diuretic peptides, which include a diuretic peptide from the Belgium forest ant *Formica polyctena* (Laenen, 1999), are highly conserved (at least 71 % sequence identity) in insects from diverse orders (Dictyoptera, Diptera and Hymenoptera), it is likely that they have an important physiological function(s).

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References

Adams, M. D. Celniker, S. E. Holt, R. A. Evans, C. A. Gocayne, J. D. Amanatides, P. G. Scherer, S. E. Li, P. W. Hoskins, R. A. Galle, R. F. et al. (2000). The genome sequence of *Drosophila melanogaster*. *Science* **287**, 2185–2195.

Bolton, A. E. (1989). Comparative methods for the radiolabelling of peptides. In *Neuroendocrine Peptide Methodology* (ed. P. M. Conn), pp. 291–401. New York: Academic Press.

Brooker, G., Harper, J. F., Terasaki, W. L. and Moylan, R. D. (1979). Radioimmunoassy of cyclic AMP and cyclic GMP. In *Advances in Cyclic Nucleotide Research*, vol. 10 (ed. G. Brooker, P. Greengard and G. A. Robinson), pp. 1–33. New York: Raven Press.

Coast, G. M. (1995). Synergism between diuretic peptides controlling ion and fluid transport in insect Malpighian tubules. *Regul. Pept.* **57**, 283–296.

Coast, G. M. and Kay, I. (1994). The effects of *Acheta* diuretic peptide on isolated Malpighian tubules from the house cricket *Acheta domesticus*. *J. Exp. Biol.* **187**, 225–243.

Davies, S. A., Huesmann, G. R., Maddrell, S. H. P., O'Donnell, M. J., Skaer, N. J. V., Dow, J. A. T. and Tublitz, N. J. (1995). CAP2b, a cardioacceleratory peptide, is present in *Drosophila* and stimulates tubule fluid secretion *via* cGMP. *Am. J. Physiol.* **269**, R1321–R1326.

Dow, J. A. T., Maddrell, S. H. P., Görtz, A., Skaer, N. J. V., Brogan, S. and Kaiser, K. (1994). The Malpighian tubules of *Drosophila melanogaster*: a novel phenotype for studies of fluid secretion and its control. *J. Exp. Biol.* **197**, 421–428.

Furuya, K., Milchak, R. J., Schegg, K. M., Zhang, J., Tobe, S. S., Coast, G. M. and Schooley, D. A. (2000). Cockroach diuretic hormones: characterization of a calcitonin-like peptide in insects. *Proc. Natl. Acad. Sci. USA* **97**, 6469–6474.

Holman, G. M., Nachman, R. J. and Coast, G. M. (1999). Isolation, characterization and biological activity of a diuretic myokinin neuropeptide from the housefly, *Musca domestica*. *Peptides* **20**, 1–10.

Kay, I., Coast, G. M., Cusinato, O., Wheeler, C. H., Totty, N. F. and Goldsworthy, G. J. (1991a). Isolation and characterization of a diuretic peptide from *Acheta domesticus* – evidence for a family of insect diuretic peptides. *Biol. Chem. Hoppe-Seyler* **372**, 505–512.

Kay, I., Patel, M., Coast, G. M., Totty, N. F., Mallet, A. I. and Goldsworthy, G. J. (1992). Isolation, characterization and biological activity of a CRF-related diuretic peptide from *Periplaneta americana*. *Regul. Peptides* **42**, 111–122.

Kay, I., Wheeler, C. H., Coast, G. M., Totty, N. F., Cusinato, O., Patel, M. and Goldsworthy, G. J. (1991b). Characterization of a diuretic peptide from *Locusta migratoria*. *Biol. Chem. Hoppe-Seyler* **372**, 929–934.

King, D. S., Fields, C. G. and Fields, G. B. (1990). A cleavage method which minimizes side reactions following FMOC solid phase peptide synthesis. *Int. J. Peptide Protein Res.* **36**, 255–266.

Laenen, B. (1999). Purification, characterization and mode of action of endogenous neuroendocrine factors in the forest ant, *Formica polyctena*. PhD thesis, Limburgs Universitair Centrum, Belgium.

Lehmberg, E., Ota, R. B., Furuya, K., King, D. S., Applebaum, S. W., Ferenz, H.-J. and Schooley, D. A. (1991). Identification of a diuretic hormone of *Locusta migratoria*. *Biochem. Biophys. Res. Commun.* **179**, 1036–1041.

Leyssens, A., Zhang, S. L., Van Kerkhove, E. and Steels, P. (1993). Both dinitrophenol and Ba^{2+} reduce KCl and fluid secretion in Malpighian tubules of *Formica polyctena*: the role of the apical H^+ and K^+ concentration gradient. *J. Insect Physiol.* **39**, 1061–1073.

Linton, S. M. and O'Donnell, M. J. (1999). Contributions of K^+:Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 1561–1570.

O'Donnell, M. J., Dow, J. A. T., Huesmann, G. R., Tublitz, N. J. and Maddrell, S. H. P. (1996). Separate control of anion and cation transport in Malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **199**, 1163–1175.

O'Donnell, M. J., Rheault, M. R., Davies, S. A., Rosay, P., Harvey, B. J., Maddrell, S. H. P., Kaiser, K. and Dow, J. A. T. (1998). Hormonally controlled chloride movement across *Drosophila* tubules is *via* ion channels in stellate cells. *Am. J. Physiol.* **274**, R1039–R1049.

Pannabecker, T. L., Hayes, T. K. and Beyenbach, K. W. (1993). Regulation of epithelial shunt conductance by the peptide leucokinin. *J. Membr. Biol.* **132**, 63–76.

Petzel, D. H., Pirotte, P. T. and Van Kerkhove, E. (1999). Intracellular and luminal pH measurements of Malpighian tubules of the mosquito *Aedes aegypti*: the effects of cAMP. *J. Insect Physiol.* **45**, 973–982.

Riegel, J. A., Farndale, R. W. and Maddrell, S. H. P. (1999). Fluid secretion by isolated Malpighian tubules of *Drosophila melanogaster* Meig.: effects of organic anions, quinacrine and a diuretic factor found in the secreted fluid. *J. Exp. Biol.* **202**, 2339–2348.

Rosay, P., Davies, S. A., Yu, Y., Sozen, A., Kaiser, K. and Dow, J. A. T. (1997). Cell-type specific calcium signalling in a *Drosophila* epithelium. *J. Cell Sci.* **110**, 1683–1692.

Schoofs, L., Holman, G. M., Proost, P., Van Damme, J., Hayes, T. K. and De Loof, A. (1992). Locustakinin, a novel myotropic peptide from *Locusta migratoria*, isolation, primary structure and synthesis. *Regul. Pept.* **37**, 49–57.

Schooley, D. A. (1993). Insect diuretic hormones with homology to sauvagine/CRF/urotensin I. In *Structure and Function of Primary Messengers in Invertebrates: Insect Diuretic and Antidiuretic Peptides*, vol. 12 (ed. K. W. Beyenbach), pp. 22–37. Basel: Karger.

Terhzaz, S., O'Connell, F. C., Pollock, V. P., Kean, L., Davies, S. A., Veenstra, J. A. and Dow, J. A. T. (1999). Isolation and characterization of a leucokinin-like peptide of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 3667–3676.

Wang, S., Rubenfeld, A. B., Hayes, T. K. and Beyenbach, K. W. (1996). Leucokinin increases paracellular permeability in insect Malpighian tubules. *J. Exp. Biol.* **199**, 2537–2542.

Wieczorek, H., Putzenlechner, M., Zeiske, W. and Klein, U. (1991). A vacuolar-type proton pump energizes K^+/H^+ antiport in an animal plasma-membrane. *J. Biol. Chem.* **266**, 15340–15347.

Wright, J. C. and O'Donnell, M. J. (1992). Osmolarity and electrolyte composition of pleon fluid in *Porcello scaber* (Crustacea, Isopoda, Oniscidea): implications for water vapour absorption. *J. Exp. Biol.* **164**, 189–203.