The Journal of Experimental Biology 210, 3979-3989
Published by The Company of Biologists 2007
doi:10.1242/jeb.006056

An antidiuretic peptide (Tenmo-ADFb) with kinin-like diuretic activity on Malpighian tubules of the house cricket, *Acheta domesticus* (L.)

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Accepted 3 September 2007

Summary

Acheta domesticus **is reported to have an antidiuretic hormone that reduces Malpighian tubule secretion. Identified peptides known to work in this way (Tenmo-ADFa and ADFb, and Manse-CAP$_{2b}$) were tested as candidates for the unidentified hormone, along with their second messenger, cyclic GMP. Only Tenmo-ADFb was active, but was diuretic, as was 8-bromo cyclic GMP. The activity of Tenmo-ADFb is comparable to that of the cricket kinin neuropeptide, Achdo-KII, but it is much less potent. Its activity was unaffected by deleting either the six N-terminal residues or the C-terminal phenylalanine.**

At high concentrations, tubule secretion is doubled by Tenmo-ADFb and Achdo-KII, but their actions are non-additive, suggesting they have a similar mode of action. Both stimulate a non-selective KCl and NaCl diuresis, which is consistent with the opening of a transepithelial Cl^- conductance. In support of this, the diuretic response to Tenmo-ADFb and Achdo-KII is prevented by a ten-fold reduction in bathing fluid chloride concentration, and both peptides cause the lumen-positive transepithelial voltage to collapse. The Cl^- conductance pathway appears likely to be transcellular, because the Cl^- channel blocker DPC reduces both basal and peptide-stimulated rates of secretion. The effects of 8-bromo cyclic GMP on transepithelial voltage and composition of the secreted fluid are markedly different from those of Tenmo-ADFb.

This is the first report of the antidiuretic factor Tenmo-ADFb stimulating tubule secretion. Although the actions of Tenmo-ADFb are indistinguishable from those of Achdo-KII, it is unlikely to act at a kinin receptor, because the core sequence (residues 7–12) lacks the Phe and Trp residues that are critical for kinin activity.

Key words: Malpighian tubule, fluid secretion, ion transport, electrophysiology, diuretic hormone, antidiuretic factor, kinin neuropeptide.

Introduction

The excretory process of insects is controlled by both diuretic and antidiuretic hormones (Coast et al., 2002a; Schooley et al., 2005). In general, diuretic hormones act on Malpighian tubules to increase secretion of primary urine, whereas antidiuretic hormones stimulate fluid reabsorption in the hindgut (ileum and rectum). There are, however, exceptions to this, with early reports of an antidiuretic factor acting on Malpighian tubules to reduce secretion of primary urine (Spring et al., 1988) and of a diuretic factor that appears to decrease fluid uptake from the hindgut (Wheelock et al., 1988).

The first identified peptide shown to reduce primary urine production was Manse-CAP$_{2b}$, which is a cardioacceleratory peptide (CAP) from the tobacco hornworm, *Manduca sexta* (Huesmann et al., 1995). Manse-CAP$_{2b}$ acts *via* cyclic GMP to reduce secretion by serotonin-stimulated Malpighian tubules of the blood-sucking bug *Rhodnius prolixus* (Quinlan et al., 1997). Surprisingly, the same peptide acts *via* cyclic GMP and Ca^{2+} to stimulate primary urine production by Malpighian tubules of the fruit fly, *Drosophila melanogaster* (Dow and Davies, 2003; Terhzaz et al., 2006). Subsequently, two other antidiuretic peptides (Tenmo-ADFa and Tenmo-ADFb) that act on Malpighian tubules have been identified from a pupal head extract of the mealworm beetle, *Tenebrio molitor*, based upon their ability to increase cyclic GMP production (Eigenheer et al., 2002; Eigenheer et al., 2003). Both peptides reduce secretion by the free (proximal) portion of Malpighian tubules from last-instar mealworm larvae (Eigenheer et al., 2002; Eigenheer et al., 2003), an effect that is mimicked by exogenous cyclic GMP. Tenmo-ADFa is extraordinarily potent in the fluid secretion assay, with an EC_{50} of 10 fmol l^{-1} compared with 240 pmol l^{-1} for Tenmo-ADFb. Manse-CAP$_{2b}$ also stimulates cyclic GMP production by *T. molitor* tubules and reduces fluid secretion, but it is considerably less potent than either of the native peptides, with an EC_{50} of 85 nmol l^{-1} (Wiehart et al., 2002).

The antidiuretic activity of Manse-CAP$_{2b}$ and the Tenmo-ADFs appears to result from the cyclic GMP-dependent activation of a cyclic AMP-specific phosphodiesterase, which will lower intracellular levels of cyclic AMP, a second messenger that stimulates diuresis (Quinlan and O'Donnell, 1998; Wiehart et al., 2002). Thus, Manse-CAP$_{2b}$ and Tenmo-ADFa antagonise the actions of diuretic hormones that use

cyclic AMP as a second messenger, namely serotonin in *R. prolixus* and a corticotropin-releasing factor (CRF)-related peptide (Tenmo-DH_{37}) in *T. molitor* (Quinlan and O'Donnell, 1998; Quinlan et al., 1997; Wiehart et al., 2002). Tenmo-ADFa has also been shown to act *via* cyclic GMP in inhibiting fluid secretion and ion (Na^+, K^+ and Cl^-) transport by Malpighian tubules of the yellow fever mosquito, *Aedes aegypti* (Massaro et al., 2004), possibly by reducing $Na^+/K^+/2Cl^-$ cotransport across the basal membrane, which is known to be activated by cyclic AMP (Hegarty et al., 1991).

The first report of an antidiuretic hormone acting on Malpighian tubules came from the observation that haemolymph from dehydrated house crickets (*Acheta domesticus*) reduced primary urine production, whereas haemolymph from rehydrated insects had the opposite effect (Spring et al., 1988). Additionally, neurosecretory material was lost from corpora cardiaca of dehydrated crickets, which is consistent with the release of an antidiuretic hormone. A factor that reduced Malpighian tubule secretion was partially purified from a methanolic extract of the corpora cardiaca but was not further characterised (Spring et al., 1988). We have therefore tested those peptides that have been shown to reduce primary urine production (Manse-CAP_{2b}, Tenmo-ADFa and Tenmo-ADFb) for effects on cricket Malpighian tubules. Of the peptides tested, only Tenmo-ADFb was active, but it had diuretic rather than antidiuretic activity. Here, we describe the actions of Tenmo-ADFb on cricket tubules and present results from an initial structure/activity study using N-terminal and C-terminal deleted analogues. We show that the actions of Tenmo-ADFb resemble those of the diuretic/myotropic *A. domesticus* kinins (Achdo-Ks), although it most likely acts at a different receptor.

Materials and methods

Insects

Crickets were reared according to methods described previously (Clifford et al., 1977) and were maintained at 28°C under a 12 h:12 h light:dark regime. They were fed a diet of wheat germ and ground cat food, with water provided *ad libitum*. The donor insects used in the current study were adult females aged between 7 and 14 days.

Fluid secretion assay

The 'Ramsay assay' used to measure fluid secretion by cricket Malpighian tubules has been described in detail elsewhere (Coast, 1988). Briefly, single tubules are transferred to small (5 μl) drops of saline beneath water-saturated paraffin oil. The saline used differed from that employed in earlier studies in that the K^+ concentration was increased at the expense of Na^+ from 8.6 mmol l^{-1} to 25.5 mmol l^{-1}, which supports a higher rate of secretion by kinin-stimulated tubules (G.M.C., unpublished observation). The composition of the saline was as follows (in mmol l^{-1}): NaCl, 100; KCl, 8.6; $CaCl_2$, 2; $MgCl_2$, 8.5; $NaHCO_3$, 2.1; $KHCO_3$, 1.9; KH_2PO_4, 4; KOH, 11; proline, 10; glucose, 24; Hepes (*N*-2-hydroxyethylpiperazine-*N*′2-ethanesulphonic acid), 25; pH adjusted to 7.2 with 1 mol l^{-1} NaOH. After a 40 min equilibration period, the bathing fluid was changed and secreted droplets removed with a fine glass rod. Thereafter, secreted droplets were removed at 15–45 min intervals before and after challenging the tubules with test compounds dissolved in fresh saline. Droplets of secreted fluid were allowed to sink onto the non-wettable base of the Petri dish and their diameter (d) measured using an ocular micrometer. Droplet volume (picolitres; pl) was calculated as $(\pi d^3)/6$, and the rate of secretion (pl mm^{-1} min^{-1}) obtained by dividing the volume by the collection period (min) and by the length of tubule (mm) within the drop of bathing fluid. Unless otherwise stated, results are presented either as the rate of secretion or as diuretic activity (Δ pl mm^{-1} min^{-1}), which is defined as the difference between rates of secretion measured before and after challenging tubules with secretagogues.

Secreted fluid analysis

Secreted fluid droplets were collected under water-saturated paraffin oil using the Ramsay assay. Samples collected over 30 min intervals before and after adding test compounds to the bathing fluid were transferred by micropipette to a second Sylgard-lined Petri dish containing water-saturated paraffin oil for analysis using ion-selective microelectrodes (Coast et al., 2001; Ianowski and O'Donnell, 2004). Separate experiments were performed for the measurement of secreted fluid pH and Cl^-, and for Na^+ and K^+, which were analysed in the same samples. Measurements of pH and Cl^- were made within 5 min of droplet collection, and Na^+ and K^+ within 20 min.

The pH electrodes were prepared using hydrogen ion exchange resin (IE 010; World Precision Instruments, Sarasota, FL, USA) and were backfilled with 0.5 mol l^{-1} citric acid containing 10 mmol l^{-1} NaCl adjusted to pH 6.0. The reference electrode was filled with 3 mol l^{-1} KCl. The K^+ electrodes were based on K^+ ionophore I, cocktail A (Fluka, Buchs, Switzerland), and backfilled with 500 mmol l^{-1} KCl. Sodium electrodes were based on Na^+ ionophore II, cocktail A (Fluka), and backfilled with 500 mmol l^{-1} NaCl. For both K^+ and Na^+ measurements, the reference electrode was filled with 1 mol l^{-1} LiCl. Chloride-sensitive electrodes were based on the Corning Cl^- exchanger 477913 (IE-173; World Precision Instruments) and backfilled with 0.5 mol l^{-1} KCl. The tip and shank of the reference electrode was filled with 3 mol l^{-1} sodium acetate, and 3 mol l^{-1} KCl was used to fill the shaft (Wright and O'Donnell, 1992). The electrodes were connected *via* Ag/AgCl half-cells to a high impedance electrometer (F-223A; World Precision Instruments), which was connected in turn to a data acquisition system (Datacan V; Sable Systems, Henderson, NV, USA).

Calibration solutions for pH microelectrodes were prepared by titration of a standard reference buffer (pH 7.0; Thermo Russell, Fife, Scotland, UK) with 1 mol l^{-1} HCl or 1 mol l^{-1} NaOH to give solutions encompassing the range pH 6 to pH 8. Potassium electrodes were calibrated in mixed solutions of 200 mmol l^{-1} KCl and 200 mmol l^{-1} NaCl, whereas Na^+ electrodes were calibrated in mixed solutions of 200 mmol l^{-1} NaCl and 200 mmol l^{-1} LiCl. Potassium is known to interfere with Na^+ measurements and this was corrected for as previously described (Ianowski and O'Donnell, 2004). Chloride electrodes were calibrated in 20–200 mmol l^{-1} KCl. Reference and ion-selective electrodes were positioned in secreted fluid droplets or calibration solutions beneath water-saturated paraffin oil, and the potential recorded once it had stabilised (after about 30 s). Electrodes were deemed acceptable if the calibration curve was

linear with a slope per decade change in ion concentration of ⩾52 mV (Na^+), ⩾54 mV (K^+), ⩾54 mV (Cl^-) or ⩾56 mV (H^+).

Measurement of transepithelial and basolateral membrane voltages

Isolated Malpighian tubules were anchored at each end within slits cut into a small block of Sylgard mounted in a custom-built chamber. Writhing movements of the tubule were restricted by putting it under slight tension. This allowed stable recordings of both transepithelial and intracellular voltages from the mid-portion of the tubule. The chamber (~250 μl volume) was perfused at 1 ml min^{-1} with the same saline that was used in the diuretic assay. Perfusion was stopped prior to the addition of test compounds and then restarted to wash-off. Microelectrodes (20–40 MΩ resistance when filled with 3 mol l^{-1} KCl) were drawn from 1 mm o.d. filament glass tubing (GC100F-75; Clark Electromedical Instruments, Pangbourne, UK) using a vertical pipette puller (PUL-100; World Precision Instruments). After backfilling with 3 mol l^{-1} KCl, they were connected to a high-impedance electrometer (M-707A; World Precision Instruments) *via* an Ag/AgCl half-cell, the circuit being completed through a KCl reference electrode (DRIREF-450; World Precision Instruments) placed in the perfusion chamber. Basal membrane (V_b) and transepithelial voltages (V_t) were measured in the main tubule segment close to where it was anchored into the Sylgard. Microelectrodes were advanced at an oblique angle using an hydraulic micromanipulator (MMO-203; Narishigi, Tokyo, Japan) until a sudden jump in potential indicated the basal membrane of a principal cell had been impaled. Recordings of V_b were accepted if the potential remained stable (±2 mV) for >30 s and returned to 0±2 mV after withdrawal of the electrode. Similar criteria were adopted when recording V_t after the microelectrode had been advanced through the apical membrane into the tubule lumen. Results were recorded digitally using a data acquisition system (Datacan V; Sable Systems). Recordings of V_t were paused during insertion of the microelectrode into the lumen, which was readily seen as a positive jump in potential.

Measurement of myotropic activity

Insect kinins have been shown to stimulate the contractile activity of the hindgut in cockroaches [*Leucophaea maderae* (Holman et al., 1986)], houseflies [*Musca domestica* (Coast et al., 2002b), *A. aegypti* (Veenstra et al., 1997) and *R. prolixus* (Te Brugge and Orchard, 2002)], which may assist the excretory process. Tenmo-ADFb and Achdo-KII were therefore tested for myotropic activity on cricket hindgut. Insects were killed by decapitation and the abdomen opened along its entire length with a mid-ventral incision. The hindgut was dissected free of tracheae and Malpighian tubules and severed just anterior to the junction of the ileum with the colon. A fine thread was tied around the short portion of ileum remaining, and the terminal abdominal segment with the hindgut attached was then cut free of the remainder of the body. The isolated hindgut (colon and rectum) was transferred to a shallow chamber (volume ~1 ml) fashioned from Sylgard and secured in place with a fine minutin pin through the cuticle of the terminal segment. The thread attached to the anterior hindgut was secured to a 10 g force transducer (WPI FORT10) coupled to a Sable Systems CP302 preamplifier. The output was recorded on a strip chart recorder or digitally using Datacan V (Sable Systems). The chamber containing the isolated hindgut was perfused continuously at 1 ml min^{-1} with cockroach hindgut saline (Cook and Holman, 1978), which, in contrast to the cricket saline used for the diuretic assay, supported regular spontaneous contractile activity. Peptides dissolved in 1 ml saline were added to the preparation by switching the perfusate between normal saline and the test solution. The peptide was immediately washed off by perfusing with normal saline after the delivery of 1 ml of the test solution.

Peptide synthesis

The synthesis of Tenmo-ADFa, Tenmo-ADFb and Manse-CAP_{2b} has been described elsewhere (Eigenheer et al., 2002; Eigenheer et al., 2003; Nachman and Coast, 2007). The Tenmo-ADFb analogs were synthesised *via* Fmoc methodology on Rink Amide resin (Novabiochem, San Diego, CA, USA) using Fmoc-protected amino acids (Advanced Chemtech, Louisville, KY, USA) on an ABI 433A peptide synthesiser (Applied Biosystems, Foster City, CA, USA) under previously described conditions (Nachman et al., 1997). Crude products were purified on a Waters C_{18} Sep-Pak cartridge and a Delta-Pak C_{18} reverse-phase column (8×100 mm, 15 mm particle size, 100 Å pore size) on a Waters 510 HPLC controlled with a Millennium 2010 chromatography manager system (Waters, Milford, MA, USA) with detection at 214 nm and run at ambient temperature. Solvent A was 0.1% aqueous trifluoroacetic acid (TFA), and Solvent B was 80% aqueous acetonitrile containing 0.1% TFA. The initial solvent consisted of 20% B and was followed by the Waters linear program to 100% B over 40 min with a flow rate of 2.0 ml min^{-1}. The Delta-Pak C_{18} retention times were: ADFb[2–13], 15.0 min; ADFb[7–13], 12.5 min; ADFb[8–13], 9.0 min; ADFb[1–12], 8.5 min. Most of the peptides were further purified on a Waters Protein Pak I125 column (7.8×300 mm) (Milligen Corp., Milford, MA, USA). Peptides were eluted under isocratic conditions, with the solvent consisting of 80% acetonitrile containing 0.01% TFA and with a flow rate of 2.0 ml min^{-1}. Retention times on the Waters Protein Pak column were: ADFb[2–13], 10.5 min; ADFb[7–13], 8.0 min; ADFb[1–12], 10.0 min. Amino acid analysis was carried out under previously reported conditions (Nachman et al., 1997) and was used to quantify the peptide and to confirm its identity. It resulted in the following analyses: ADFb[2–13]: Asp[1.0], Phe[1.0], Gly[2.3], His[1.2], Ile[0.8], Lys[0.9], Tyr[2.0]; ADFb[7–13]: Phe[1.0], Gly[0.9], His[1.0], Ile[0.9], Lys[0.9], Pro[0.9], Tyr[1.3]; ADFb[8–13]: Phe[1.0], Gly[1.1], His[0.7], Ile[0.7], Pro[1.1], Tyr[0.9]; ADFb[1–12]: Asp[1.6], Gly[1.9], His[1.0], Ile[0.9], Lys[1.0], Ser[1.0], Tyr[2.0]. The identities of the peptide analogues were confirmed *via* MALDI-TOF-MS on a Kratos Kompact Probe MALDI-TOF MS machine (Kratos Analytical, Ltd, Manchester, UK) with the presence of the following molecular ions ($M+H^+$): ADFb[2–13], 1397.0 [$M+H^+$]; ADFb[7–13], 860.9 [$M+H^+$]; ADFb[8–13], 733.5 [$M+H^+$]; ADFb[1–12], 1415.5 [$M+H^+$].

Chemicals

The chloride channel blockers diphenylamine-2-carboxylate (DPC) and 5-nitro-2-(3-phenylpropylamino)-benzoic acid

(NPPB) were obtained from Calbiochem (Merck Biosciences Ltd, Beeston, UK) and were prepared as stock solutions in ethanol and dimethyl sulfoxide, respectively. All other chemicals were obtained from Sigma-Aldrich (Poole, Dorset, UK).

Calculations

Net electrochemical gradients ($\Delta\mu/F$, in mV) across the epithelium for K^+, Na^+, H^+ and Cl^- were calculated as described previously (O'Donnell et al., 1996) using the equation:

$$\Delta\mu/F = (\boldsymbol{R}T/F) \ln (a_L/a_{BS}) + zV_t = 59\log (a_L/a_{BS}) + zV_t ,$$

where z is the valency, a_L is the activity in the lumen (mmol l^{-1}), a_{BS} is the activity in the bathing fluid, $\boldsymbol{R}$ is the universal gas constant, T is the absolute temperature (K) and F is the Faraday constant. In practice, ion concentrations rather than activities were used, on the assumption that activity coefficients in the tubule fluid were likely to be similar to those in the bathing fluid. When $\Delta\mu/F$ is zero, the ion is in equilibrium across the epithelium, whereas a positive value of $\Delta\mu/F$ means that the luminal ion concentration exceeds the equilibrium value, i.e. active transport. A negative value for $\Delta\mu/F$ indicates that the luminal ion concentration is below equilibrium and net passive diffusion from bath to lumen is favoured.

Data are presented as means ± s.e.m. for the number of determinations indicated (N). Tests for significance were performed with GraphPad Instat 3.06 (GraphPad Software, San Diego, CA, USA) using paired and unpaired Student t-tests as appropriate. Differences were considered significant when $P<0.05$. Dose–response curves with variable slope were fitted using Prism™ v. 4.02 (GraphPad Software).

Results

Effect of putative antidiuretic peptides on fluid secretion

In a preliminary screen, Manse-CAP_{2b}, Tenmo-ADFa and Tenmo-ADFb were each tested at 1 μmol l^{-1} for effects on fluid secretion by cricket tubules. The membrane-permeant cyclic GMP analogue, 8-bromo cyclic GMP (1 mmol l^{-1}), was also screened for activity since the cyclic nucleotide acts as a second messenger for all three peptides. The cricket diuretic kinin neuropeptide, Achdo-KII, was included in the assay as a positive control. The results presented in Fig. 1 show that, of the three putative antidiuretic peptides, only Tenmo-ADFb was active, but it stimulated rather than inhibited tubule secretion. Indeed, the diuretic activity (defined as the increase in rate of secretion over that measured in the control period; Δ pl mm^{-1} min^{-1}) of Tenmo-ADFb (Δ 191±32 pl mm^{-1} min^{-1}; N=9) was not significantly different (P=0.167; unpaired t-test) from that of Achdo-KII (Δ 250±26 pl mm^{-1} min^{-1}; N=13). Exogenous 8-bromo cyclic GMP also produced a small, but significant ($P \ll 0.001$; paired t-test), increase in fluid secretion (Δ 60±9 pl mm^{-1} min^{-1}; N=10).

A dose–response curve for the diuretic activity of Tenmo-ADFb is shown in Fig. 2A. Data are expressed as percentages of the response to a supramaximal dose (1 nmol l^{-1}) of Achdo-KII

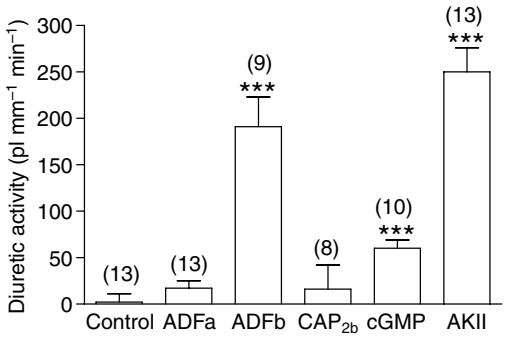

Fig. 1. Peptides known to reduce primary urine production [Tenmo-ADFa (ADFa), Tenmo-ADFb (ADFb) and Manse-CAP_{2b} (CAP_{2b})] were tested at 1 μmol l^{-1} for an effect on cricket tubules, along with their second messenger cyclic GMP [1 mmol l^{-1} 8-bromo cyclic GMP (cGMP)]. The cricket kinin Achdo-KII (1 nmol l^{-1}; AKII) was included in the assay as a positive control. Bars represent mean values for the change in rate of secretion (Δ pl mm^{-1} min^{-1}) following addition of test compounds, and vertical lines represent + 1 s.e.m. for the number of replicates indicated in parentheses. Tenmo-ADFb and 8-bromo cyclic GMP significantly increased (***P<0.001) fluid secretion, and the response to the former was comparable to that of Achdo-KII.

assayed alongside Tenmo-ADFb on tubules removed from the same insect. The apparent EC_{50} is 1.5 μmol l^{-1}, with 95% confidence limits of 0.8–2.8 μmol l^{-1}, and the best fit value for the top of the curve is 91±8% of the response to Achdo-KII. A dose–response curve for Achdo-KII in the same high K^+ saline is shown in Fig. 2B. The apparent EC_{50} is 10.2 pmol l^{-1}, with 95% confidence limits of 0.7–148.7 pmol l^{-1}. This compares with an EC_{50} of 22 pmol l^{-1} for Achdo-KII when assayed in cricket saline containing 9.6 mmol l^{-1} K^+ (Coast et al., 1990).

Structure–activity studies

Several analogues of Tenmo-ADFb were tested for diuretic activity and the results are summarised in Table 1. The deletion

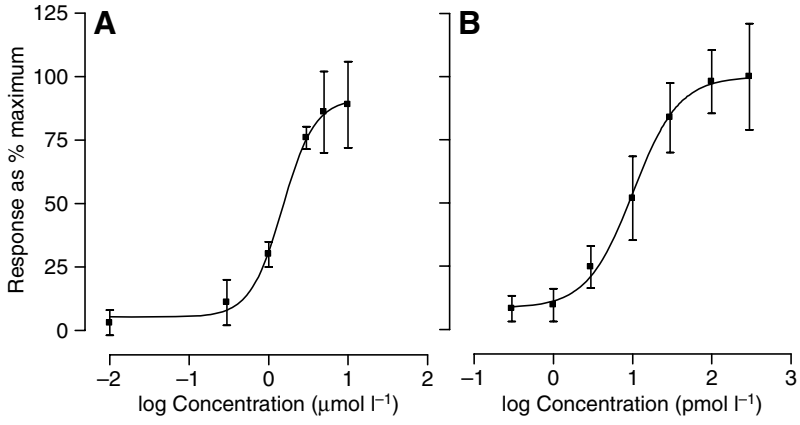

Fig. 2. Dose–response curves for the diuretic activity of (A) Tenmo-ADFb and (B) Achdo-KII. Results are expressed as a percentage of the response to a supramaximal concentration (1 nmol l^{-1}) of Achdo-KII. Data points are the means ± 1 s.e.m. of 7–10 replicates. Note the vast difference in potency between Tenmo-ADFb (EC_{50} 1.5 μmol l^{-1}) and Achdo-KII (EC_{50} 10.2 pmol l^{-1}).

Table 1. *The diuretic activity and potency of N- and C-terminal deletion analogues of Tenmo-ADFb*

Sequence	Analogue	EC_{50} (μmol l^{-1})	95% CL (μmol l^{-1})	Response maximum (%)
YDDGSYKPHIYGF-OH	ADFb	0.59	0.44–0.80	90±9
DDGSYKPHIYGF-OH	ADFb[2–13]	0.79	0.40–1.54	87±8
KPHIYGF-OH	ADFb[7–13]	0.14	0.07–0.32	92±8
PHIYGF-OH	ADFb[8–13]	Not active	Not active	
YDDGSYKPHIYG -OH	ADFb[1–12]	0.50	0.06–4.41	87±11

of six residues from the N-terminus had no effect on activity. Indeed, Tenmo-ADFb[7–13] was more potent (P<0.05) than the parent compound. However, with the removal of one further amino acid, there was a complete loss of activity, and Tenmo-ADFb[8–13] had no effect on tubule secretion at 10 μmol l^{-1}, which was the highest concentration tested. One C-terminal truncated peptide (Tenmo-ADFb[1–12]) was also tested and shown to retain diuretic activity with a potency comparable to that of the parent compound.

Comparing the activities of Tenmo-ADFb and Achdo-KII

Although Achdo-KII is considerably more potent than Tenmo-ADFb, their diuretic activity appears similar. This is further illustrated in Fig. 3, which shows the time course of the response to the two peptides when tested at supramaximal concentrations (10 μmol l^{-1} and 1 nmol l^{-1}, respectively) first separately and then together. The data are normalised by expressing as percentages of the unstimulated rate of secretion measured at 45 min. Fluid secretion increases by ~75% within 15 min of peptide addition, and the effects of Tenmo-ADFb and Achdo-KII are not additive.

Effect of Tenmo-ADFb, Achdo-KII and 8-bromo cyclic GMP on tubule fluid composition

Fig. 4 compares the pH and the Na^+, K^+ and Cl^- concentrations of tubule fluid collected over 30 min intervals before and after the addition of 10 μmol l^{-1} Tenmo-ADFb, 1 nmol l^{-1} Achdo-KII and 1 mmol l^{-1} 8-bromo cyclic GMP. Cricket tubules secrete K^+-rich urine, and the $[Na^+]$:$[K^+]$ ratio before and after the addition of Tenmo-ADFb (0.22±0.04 and 0.18±0.03; N=14; P=0.218, paired t-test) and Achdo-KII (0.23±0.02 and 0.22±0.02; N=15; P=0.417, paired t-test) did not change significantly, although both peptides produced small, but significant, increases in K^+ concentration (Fig. 4). In marked contrast, the addition of 8-bromo cyclic GMP caused the urine $[Na^+]$:$[K^+]$ ratio to virtually double from 0.23±0.06 to 0.47±0.09 (N=9; P<0.001), reflecting a significant increase in the concentration of Na^+ and a corresponding decrease in K^+ (Fig. 4).

All three secretagogues caused a small increase in Cl^- concentration (Fig. 4), but the effect of 8-bromo cyclic GMP was not quite significant (P=0.054). The secreted fluid was slightly more acidic after the addition of Tenmo-ADFb (Δ –0.05±0.02 pH units; N=18; P<0.05, paired t-test) and Achdo-KII (Δ –0.08±0.02 pH units; N=16; P<0.01, paired t-test), whereas it was significantly more alkaline in tubules challenged with 8-bromo cyclic GMP (Δ +0.18±0.03 pH units; N=10; P<0.001, paired t-test).

Diuretic activity is chloride dependent

Kinins stimulate tubule secretion by opening a Cl^- conductance pathway, which increases net transport of KCl and NaCl into the lumen along with osmotically obliged water (Beyenbach, 2003b). To determine the Cl^- dependency of the responses to Tenmo-ADFb and Achdo-KII, tubules were isolated in standard saline (controls) and in low Cl^- saline (one-tenth the normal concentration, with gluconate salts replacing chloride). After a 40 min equilibration period, fluid secretion was measured over 40 min periods before and after the addition of either 10 μmol l^{-1} Tenmo-ADFb or 1 nmol l^{-1} Achdo-KII in standard and in low Cl^- saline. Finally, all tubules were transferred to standard saline containing the test peptides, and secretion measured over a third 40 min period. The results are presented in Fig. 5. The rate of secretion by tubules held in low Cl^- saline is ~25% that of tubules in normal saline and they do not respond to the addition of either Tenmo-ADFb or Achdo-KII. When these tubules are transferred to normal saline, fluid secretion returns to levels comparable with those of the peptide-stimulated controls.

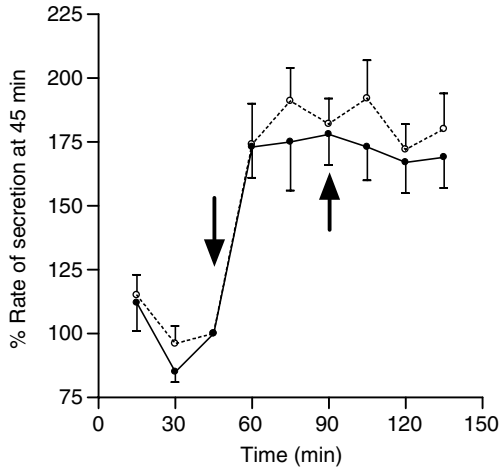

Fig. 3. Time course for the stimulation of fluid secretion by supramaximal concentrations of Tenmo-ADFb (10 μmol l^{-1}; solid symbols and line) and Achdo-KII (1 nmol l^{-1}; open symbols and dotted line). Results are normalised by being expressed as a percentage of the unstimulated rate of secretion at 45 min. Data points are the means ± 1 s.e.m. of 11–12 replicates. Fluid secretion increases rapidly following addition of the individual peptides (downward arrow) and is not further elevated when they are applied together (upward arrow).

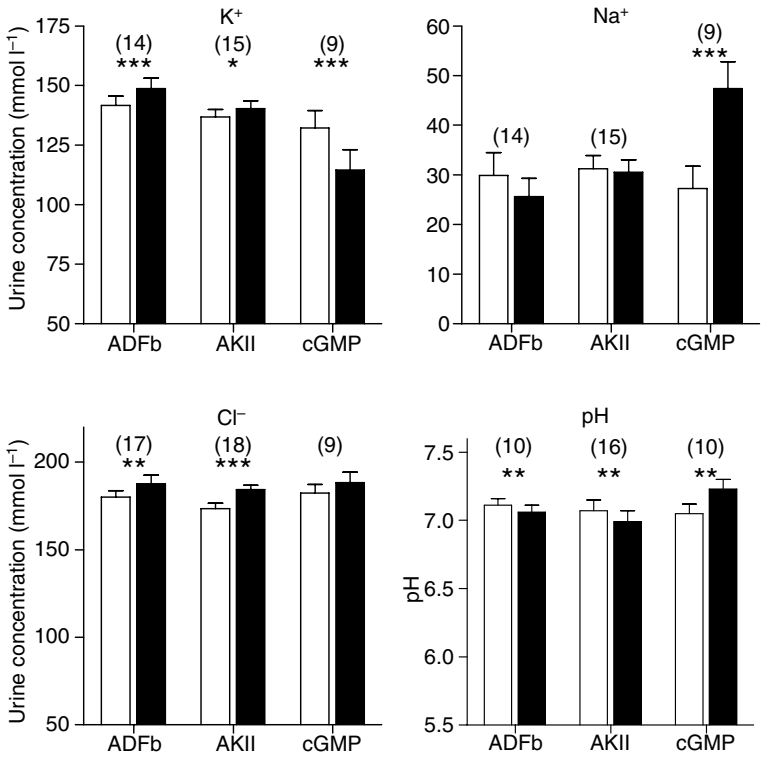

Fig. 4. Secreted fluid pH and concentrations of K^+, Na^+ and Cl^- before (open bars) and after (solid bars) the addition of 10 μmol l^{-1} Tenmo-ADFb (ADFb), 1 nmol l^{-1} Achdo-KII (AKII) and 1 mmol l^{-1} 8-bromo cyclic GMP (cGMP). Bars represent the means + 1 s.e.m. for the number of replicates shown in parentheses. Asterisks indicate where the change in composition following addition of the secretagogue is significant in a paired *t*-test; **P*<0.05; ***P*<0.01; ****P*<0.001.

Effect of chloride channel blockers on fluid secretion

Fluid secretion by unstimulated and kinin-stimulated Malpighian tubules of *D. melanogaster* is inhibited by DPC, which blocks chloride channels in vertebrate epithelial cells (O'Donnell et al., 1998). This suggests the involvement of chloride channels in transepithelial Cl^- secretion, which likely follows a transcellular route. A preliminary experiment showed that at high concentrations (2 mmol l^{-1}), DPC almost completely inhibited secretion by unstimulated cricket tubules, whereas 0.2 mmol l^{-1} DPC reduced secretion by ~50%, and this concentration was selected for testing the effect of the channel blocker on the activity of 10 μmol l^{-1} Tenmo-ADFb and 1 nmol l^{-1} Achdo-KII. Compared with controls (saline containing 0.1% ethanol), secretion by unstimulated tubules was reduced in the presence of 0.2 mmol l^{-1} DPC but increased significantly (*P*<0.001; paired *t*-test) in the presence of either Tenmo-ADFb or Achdo-KII (Fig. 6). Rates of secretion by peptide-stimulated tubules were, however, reduced significantly by the chloride channel blocker. We also tested another chloride channel blocker, NPPB, for its effect on the response to 10 μmol l^{-1} Tenmo-ADFb. At the concentration used (10 μmol l^{-1}), NPPB had no effect on basal secretion, but significantly (*P*<0.01, unpaired *t*-test) reduced secretion by peptide-stimulated tubules from 530±40 pl mm^{-1} min^{-1} (*N*=8) in control saline containing 0.1% DMSO to 345±44 pl mm^{-1} min^{-1} (*N*=10) in the presence of the channel blocker.

Tenmo-ADFb acts synergistically with 8-bromo-cyclic AMP

Synergism between kinins and cyclic AMP has been described in several insects, notably *A. domesticus* (Coast et al., 1990), *Locusta migratoria* (Coast, 1995) and *M. domestica* (Holman et al., 1999), and can be attributed to the separate activation of anion and cation transport processes, respectively. To investigate possible synergism between Tenmo-ADFb and 8-bromo-cyclic AMP, tubules were challenged with each of the secretagogues separately and together. The cyclic nucleotide was used at 10 μmol l^{-1} and Tenmo-ADFb was tested at 0.3 μmol l^{-1}. The data presented in Fig. 7A show the change in fluid secretion (Δ pl mm^{-1} min^{-1}) in the 35 min period following addition of the secretagogues. The sum of the separate effects of cyclic AMP and Tenmo-ADFb is significantly less than the response obtained when they are tested together (*P*<0.001; unpaired *t*-test), which provides evidence of synergism. Synergism could not be demonstrated between 8-bromo cyclic GMP and either Tenmo-ADFb or Achdo-KII (data not shown). However, 1 mmol l^{-1} 8-bromo cyclic GMP significantly increased secretion by tubules that were already maximally stimulated with 10 μmol l^{-1} Tenmo-ADFb (*N*=11; *P*<0.001, paired *t*-test) and 1 nmol l^{-1} Achdo-KII (*N*=11; *P*<0.001, paired *t*-test) (Fig. 7B).

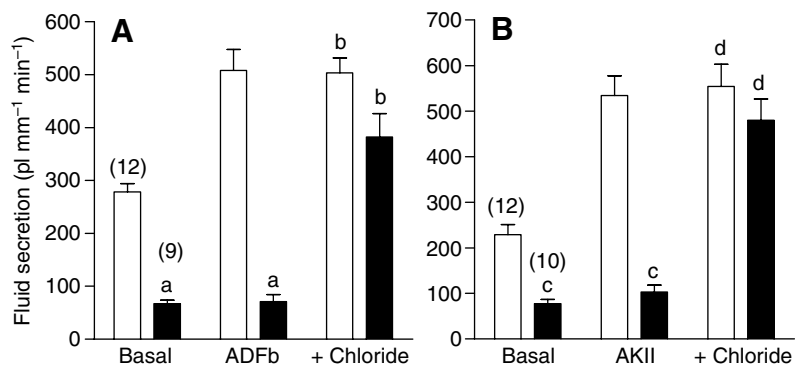

Fig. 5. Tenmo-ADFb (1 μmol l^{-1}; ADFb) and Achdo-KII (1 nmol l^{-1}; AKII) have no effect on fluid secretion by tubules incubated in low Cl^- saline (one-tenth normal [Cl^-]). Bars represent the means + 1 s.e.m. of fluid secretion by tubules in normal (open bars) and in low Cl^- (solid bars) saline. The number of replicates is shown in parentheses. In the final experimental period, all tubules were moved to normal saline (+ chloride). Note that identical letters indicate mean values that do not differ significantly i.e. *P*<0.05.

Effects on tubule electrophysiology

Kinins depolarise the transepithelial voltage (V_t) in Malpighian tubules of *A. aegypti* (Hayes et al., 1989) and *D. melanogaster* (O'Donnell et al., 1996), and we therefore determined whether Tenmo-ADFb and Achdo-KII had a similar effect on cricket tubules. The V_t of unstimulated tubules, measured with an electrode positioned in the lumen, generally oscillated by about ±10 mV (Fig. 8). Similar oscillations have been reported in Malpighian tubules of *A. aegypti* (Beyenbach et al., 2000) and *D. melanogaster* (Blumenthal, 2001) and have been attributed to rhythmic changes in transepithelial chloride conductance. The mean value of V_t was 42.9±3.4 mV (N=20) lumen positive, which is substantially higher than the value of 0.7 mV reported previously (Coast and Kay, 1994) using electrodes placed in the secreted droplet and in the bathing fluid, a method that is known to be subject to artefact (Aneshansley et al., 1988; Isaacson and Nicolson, 1989). Addition of either 10 μmol l^{-1} Tenmo-ADFb or 1 nmol l^{-1} Achdo-KII resulted in an immediate collapse of V_t, although it remained non-zero (Fig. 8A,B). The mean change in V_t following the addition of Tenmo-ADFb was –31.2±4.1 mV (N=7), which was not significantly different (P=0.507; unpaired t-test) from the effect of Achdo-KII (–28.2±4.1 mV; N=13).

In marked contrast to the effects of Tenmo-ADFb and Achdo-KII, the addition of 1 mmol l^{-1} 8-bromo cyclic GMP resulted in a significant increase in V_t (Fig. 8C); the mean change in voltage was +9.1±0.9 mV (N=7; P<0.001, paired t-test). Subsequent addition of either 10 μmol l^{-1} Tenmo-ADFb (Fig. 8C) or 1 nmol l^{-1} Achdo-KII (data not shown) caused V_t to collapse even in the continued presence of 8-bromo cyclic GMP.

Fig. 9A is a representative recording that shows the effect of the chloride channel blocker DPC on V_t. Following the addition of 0.2 mmol l^{-1} DPC, there is an immediate decrease in V_t, which then slowly recovers even in the continued presence of the channel blocker, although it never returned to its initial value. The transepithelial voltage continued to oscillate in the presence of DPC, but the oscillations were generally of reduced amplitude. DPC did not prevent the collapse of V_t following the addition of 10 μmol l^{-1} Tenmo-ADFb, but the voltage change was reduced to –23.7±2.5 mV (N=7). However, this was not significantly different (P=0.094, unpaired t-test) from the voltage change produced by Tenmo-ADFb (–31.7±3.6 mV; N=4) under control conditions (saline containing 0.1% ethanol).

Fig. 9B is a representative recording showing the effect of 10 μmol l^{-1} Tenmo-ADFb and 1 nmol l^{-1} Achdo-KII on the voltage across the principal cell basal membrane (V_b). The mean value of V_b in unstimulated tubules was –41.4±0.8 mV (N=28), and the apical membrane voltage (V_a=V_t–V_b) is therefore ~84 mV lumen positive. The oscillations seen in V_t were not observed in recordings of V_b, which reflects what has been described in *D. melanogaster* tubules (Blumenthal, 2001) and has been attributed to the low resistance of the basal membrane. The change in V_b following the addition of either peptide was less than ±2 mV.

Transepithelial electrochemical gradients for K^+, Na^+, H^+ and Cl^-

Net transepithelial electrochemical gradients ($\Delta\mu/F$) for each of the measured ions can be calculated from their respective concentrations in the bathing medium and secreted fluid (data from Fig. 4) and from measurements of V_t under basal (unstimulated) conditions and following the addition of 10 μmol l^{-1} Tenmo-ADFb, 1 nmol l^{-1} Achdo-KII or 1 mmol l^{-1} 8-bromo cyclic GMP (Table 2). Values for $\Delta\mu/F$ are necessarily approximate, because ion concentrations and transepithelial voltages were measured in different sets of tubules.

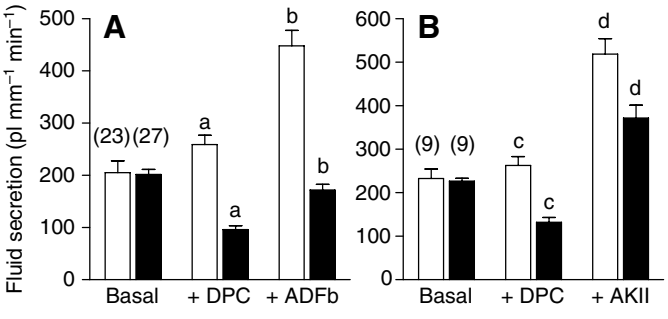

Fig. 6. The chloride channel blocker DPC depresses basal secretion and reduces the effect of (A) 10 μmol l^{-1} Tenmo-ADFb (+ADFb) and (B) 1 nmol l^{-1} Achdo-KII (+AKII). Bars represent the means + 1 s.e.m. for the number of replicates shown in parentheses. Tubules were held in control saline (open bars) or moved to a saline containing 0.2 mmol l^{-1} DPC in the first experimental period (+DPC; solid bars). Identical letters indicate significant differences between mean rates of secretion (P<0.01).

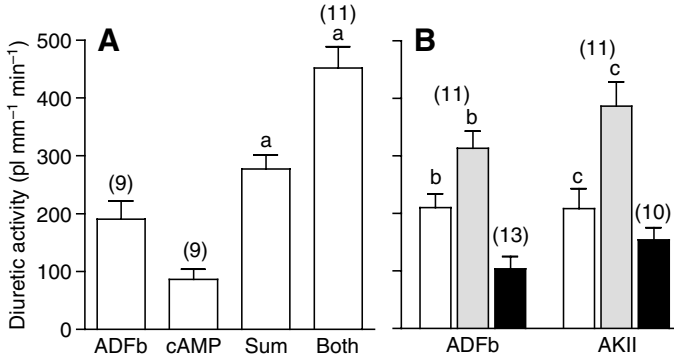

Fig. 7. (A) Evidence of synergism between 10 μmol l^{-1} 8-bromo cyclic AMP (cAMP) and 0.3 μmol l^{-1} Tenmo-ADFb (ADFb). Bars represent the means + 1 s.e.m. for the change in rate of secretion (Δ pl mm^{-1} min^{-1}) following addition of the secretagogues individually and together. The number of replicates is shown in parentheses, and identical letters indicate values that differ significantly (P<0.001). The sum of the separate responses to 8-bromo cyclic AMP and Tenmo-ADFb (Sum) is significantly less than when they are tested together (Both). (B) 8-bromo cyclic GMP (1 mmol l^{-1}) increases secretion by tubules that have been maximally stimulated by Tenmo-ADFb (10 μmol l^{-1}) and Achdo-KII (1 nmol l^{-1}; AKII). Bars represent the means + 1 s.e.m. for the change in rate of secretion (Δ pl mm^{-1} min^{-1}) after addition of the peptides alone (open bars) and then together with 8-bromo cyclic GMP (grey bars). Black bars show the diuretic activity of 1 mmol l^{-1} 8-bromo cyclic GMP alone measured in separate groups of tubules. The number of replicates is shown in parentheses, and identical letters indicate values that differ significantly (P<0.001).

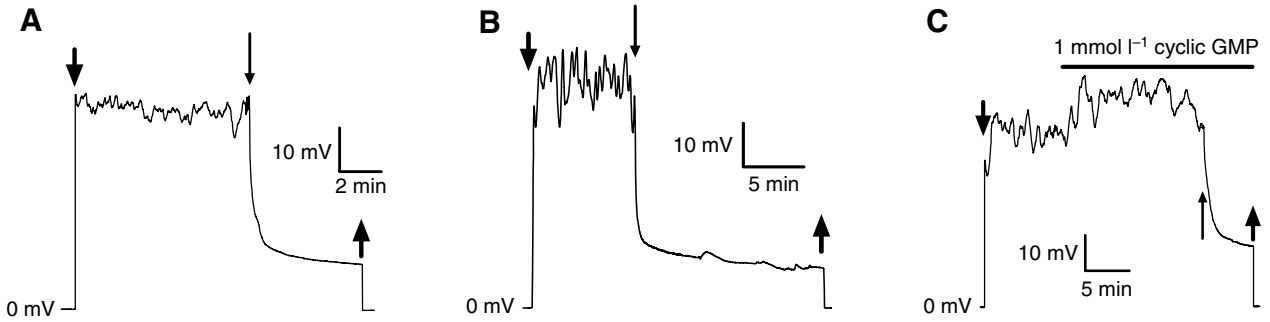

Fig. 8. Representative recordings showing the change in transepithelial voltage (V_t) following the addition of (A) 10 μmol l^{-1} Tenmo-ADFb, (B) 1 nmol l^{-1} Achdo-KII and (C) 1 mmol l^{-1} 8-bromo cyclic GMP. Thick arrows show the time of insertion (downward) and withdrawal (upward) of the microelectrode, and thin arrows show when peptides were added. The horizontal bar in C shows when 8-bromo cyclic GMP was present in the perfusate, and the thin arrow indicates the time of addition of 10 μmol l^{-1} Tenmo-ADFb.

For cations (Na^+, K^+ and H^+), $\Delta\mu/F$ is invariably positive under basal conditions (Table 2), indicating that their concentration in the secreted fluid exceeds equilibrium values. The transepithelial transport of these ions must therefore be active. Following stimulation with either Tenmo-ADFb or Achdo-KII, the magnitude of $\Delta\mu/F$ decreases, reflecting the collapse of V_t, but remains positive for K^+ and H^+, while falling to negative values for Na^+. In contrast, $\Delta\mu/F$ is either unchanged (H^+) or increases (Na^+ and K^+) in tubules stimulated with 8-bromo cyclic GMP. This is particularly marked for Na^+, with $\Delta\mu/F$ increasing from 9.1 mV to 32.3 mV after addition of the cyclic nucleotide due to increases in both V_t and the concentration of Na^+ in the luminal fluid.

The calculated net electrochemical potential for Cl^- is invariably negative and hence favours passive movement of Cl^- from the bath into the lumen. The electrochemical potential is reduced after stimulation with either Tenmo-ADFb or Achdo-KII, which cause V_t to collapse, but is increased in the presence of 8-bromo cyclic GMP.

Effect of Tenmo-ADFb and Achdo-KII on hindgut contractions

Given the similar effects of Tenmo-ADFb and Achdo-KII on tubule secretion, both peptides were tested for myotropic activity on cricket hindgut. The hindgut of *A. domesticus* contracts spontaneously when bathed in cockroach saline, and typical recordings are presented in Fig. 10, which shows also the effect of challenging the same preparation with 10 μmol l^{-1} Tenmo-ADFb (Fig. 10A), 2 nmol l^{-1} Achdo-KII (Fig. 10B) and with saline alone (Fig. 10C). Achdo-KII has a pronounced effect on the frequency and amplitude of hindgut contractions, whereas Tenmo-ADFb and saline alone were without effect. Tested on five different hindgut preparations, the percentage change in contraction frequency over 2 min intervals before and after the addition of 10 μmol l^{-1} Tenmo-ADFb was –0.4±1.6%, compared with –1.4±1.5% after adding saline, and a 47.8±4.3% increase with 3 nmol l^{-1} Achdo-KII. The threshold concentration of Achdo-KII needed to produce a readily observable effect on the frequency and/or amplitude of hindgut contractions was 1.43±0.39 nmol l^{-1} (N=12).

Discussion

Activities of identified antidiuretic factors on cricket tubules

Spring et al. reported the presence of an antidiuretic factor in *A. domesticus* that reduced Malpighian tubule secretion (Spring et al., 1988). Three unrelated peptides, Manse-CAP_{2b}, Tenmo-ADFa and Tenmo-ADFb, have since been shown to reduce tubule

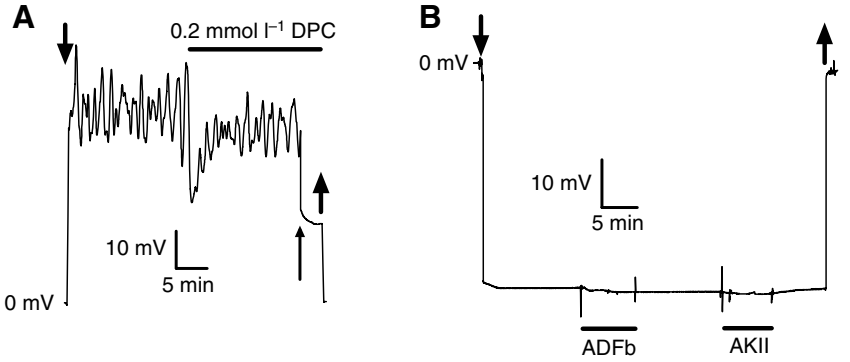

Fig. 9. (A) Representative recording of the transepithelial voltage (V_t) before and after the addition of 0.2 mmol l^{-1} diphenylamine-2-carboxylate (DPC) to the bathing saline. DPC briefly depolarises V_t but does not inhibit the spontaneous voltage oscillations. Nor does it prevent the reduction of V_t by 10 μmol l^{-1} Tenmo-ADFb (thin arrow; ADFb). (B) A representative recording of the basal membrane voltage (V_b) showing this is not affected by the addition of either 10 μmol l^{-1} Tenmo-ADFb or 1 nmol l^{-1} Achdo-KII (AKII). Thick arrows mark the point of insertion (downward) and withdrawal (upward) of the microelectrode, and horizontal bars show when peptides were present in the perfusate.

Table 2. *Net transepithelial electrochemical potentials ($\Delta\mu/F$) for Na^+, K^+, H^+ and Cl^- before and after the addition of 10 μmol l^{-1} Tenmo-ADFb, 1 nmol l^{-1} Achdo-KII or 1 mmol l^{-1} 8-bromo cyclic GMP*

	$\Delta\mu/F$ (mV)					
	Tenmo-ADFb		Achdo-KII		8-bromo cyclic GMP	
	Basal	Stimulated	Basal	Stimulated	Basal	Stimulated
Na^+	11.4	–23.7	12.5	–17.0	9.1	32.3
K^+	86.8	56.9	85.9	57.7	85.1	90.5
H^+	48.9	20.7	51.3	27	52.5	51.0
Cl^-	–34.5	–2.2	–35.4	–5.0	–34.2	–42.4

Calculations are based upon measured concentrations in the secreted urine and on mean values for the transepithelial voltage (V_t).

secretion by a cyclic GMP-dependent mechanism (Quinlan et al., 1997; Wiehart et al., 2002), and it is possible that one of them is an orthologue of the uncharacterised antidiuretic factor from *A. domesticus*. However, when tested at 1 μmol l^{-1}, none of these peptides reduced secretion by cricket tubules; neither did 1 mmol l^{-1} exogenous 8-bromo-cyclic GMP. On the other hand, Tenmo-ADFb stimulated secretion to approximately the same extent as a kinin neuropeptide, Achdo-KII, although it was about five orders of magnitude less potent in the diuretic assay (EC_{50} values of 1.5 μmol l^{-1} and 10.2 pmol l^{-1}, respectively).

Comparing the actions of Tenmo-ADFb with those of Achdo-KII and 8-bromo cyclic GMP

Data from the present study show that Tenmo-ADFb and Achdo-KII have identical effects on cricket tubules. When tested at supramaximal concentrations, they increase secretion by about 75% and their activities are non-additive, which suggests they share the same mode of action. Consistent with this suggestion, neither peptide has any effect on the $[Na^+]$:$[K^+]$ ratio of the secreted fluid, and both cause a small but significant decrease in pH. Moreover, in common with the effect of kinins on Malpighian tubules from *A. aegypti* and *D. melanogaster* (Hayes et al., 1989; O'Donnell et al., 1996), both Tenmo-ADFb and Achdo-KII cause the lumen-positive transepithelial voltage to collapse, although it remains non-zero. Kinins are known to act synergistically with exogenous cyclic AMP (Coast, 1995; Coast et al., 1990; Holman et al., 1999), and this has now been demonstrated with Tenmo-ADFb, which further supports the kinin-like actions of this antidiuretic factor from *T. molitor*.

Tenmo-ADFb acts *via* a cyclic GMP-dependent mechanism in reducing secretion by *T. molitor* tubules (Eigenheer et al., 2003), and the same second messenger could be implicated in its diuretic activity in crickets, where exogenous 8-bromo cyclic GMP stimulates tubule secretion. However, this is not consistent with the data, which show significant differences between the effects that 8-bromo cyclic GMP and Tenmo-ADFb (and Achdo-KII) have on the composition of the secreted fluid and the transepithelial voltage. Notably, 8-bromo cyclic GMP elevates the lumen-positive transepithelial voltage, doubles the $[Na^+]$:$[K^+]$ ratio of the secreted fluid and increases its pH. Moreover, the cyclic nucleotide analogue accelerates secretion by tubules that are already maximally stimulated by Tenmo-ADFb (and Achdo-KII), which indicates it has a different mode of action.

Although Tenmo-ADFb and Achdo-KII have identical effects on cricket tubules, only the latter stimulated contractions by the hindgut. It is worth noting, however, that the threshold concentration for Achdo-KII activity in the hindgut assay (1.43 nmol l^{-1}) is about 140-fold higher than its EC_{50} in the diuretic assay. If the same difference in potency were to apply to Tenmo-ADFb, then the threshold concentration for an observable effect in the myotropic assay would be about 200 μmol l^{-1}, which is 20 times higher than the maximum concentration tested.

Effect on Cl^- conductance

The generally accepted model for the diuretic activity of insect kinins is that they open a transepithelial conductance pathway for chloride, which accelerates its movement into the Malpighian tubule lumen down a favourable transepithelial electrochemical gradient. The movement of additional Cl^- into the lumen causes the lumen-positive transepithelial voltage to collapse and results

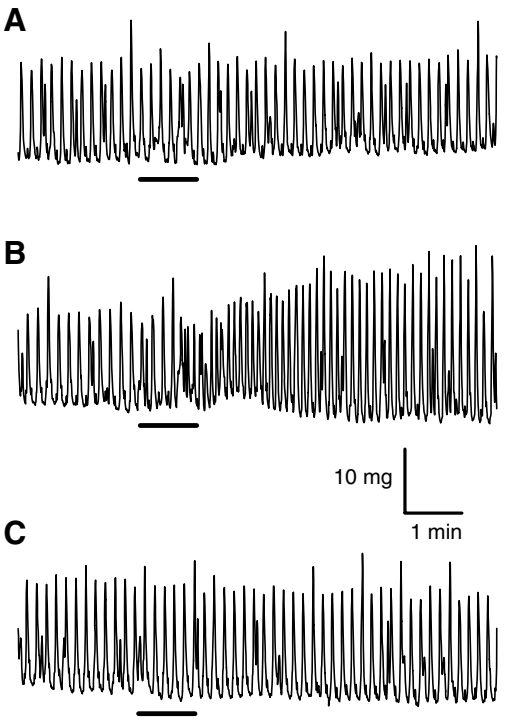

Fig. 10. A representative recording of spontaneous contractions by a cricket hindgut preparation challenged with first 10 μmol l^{-1} Tenmo-ADFb (A), then 2 nmol l^{-1} Achdo-KII (B) and finally with saline alone (C). Bars show when test substances were present in the perfusate.

in a non-selective increase in NaCl and KCl secretion accompanied by osmotically obliged water. Evidence from the present study is consistent with Tenmo-ADFb and Achdo-KII acting in a similar manner. They have an insignificant effect on the $[Na^+]$:$[K^+]$ ratio of tubule fluid, their diuretic activity is chloride dependent, as evidenced by a failure to stimulate secretion by tubules bathed in saline containing one-tenth the normal chloride concentration, and both cause V_t to collapse. Moreover, the calculated net electrochemical driving force for Cl^- ($\Delta\mu/F_{Cl}$) favours passive diffusion from bath to lumen both before and after stimulation by Tenmo-ADFb and Achdo-KII (Table 2) despite the fall in V_t. Following peptide stimulation, $\Delta\mu/F_{Cl}$ declines by ~31 mV while there is a 75% increase in net transepithelial Cl^- transport (the product of the Cl^- concentration in the tubule fluid and the rate of secretion). It follows that Tenmo-ADFb and Achdo-KII must promote a substantial increase in the Cl^- permeability (conductance) of the epithelium.

Location of the chloride conductance pathway

The Cl^- conductance pathway in the Malpighian tubules of dipteran insects lies outside of the principal cells, but there is some debate as to its precise location. In *A. aegypti*, kinins are believed to act on principal cells and to open a paracellular conductance pathway, which would require rapid remodelling of septate junctional complexes (Beyenbach, 2003a). On the other hand, in *D. melanogaster*, kinins act on a second cell type, the stellate cell, to open a transcellular Cl^- conductance pathway (O'Donnell et al., 1998; Radford et al., 2002). The Malpighian tubules of *A. domesticus* lack stellate cells (Hazelton et al., 1988), and the Cl^- conductance pathway must therefore be through either principal cells or septate junctional complexes, as in *A. aegypti*.

Results obtained with the epithelial chloride channel blockers DPC and NPPB are consistent with Tenmo-ADFb and Achdo-KII acting to open a transcellular Cl^- conductance pathway, i.e. through the principal cells. Thus, fluid secretion by peptide-stimulated tubules was significantly reduced by DPC (Tenmo-ADFb and Achdo-KII) and NPPB (Tenmo-ADFb), while DPC also decreased the extent to which V_t fell in response to Tenmo-ADFb, although the difference (8 mV) was not significant. It is worth noting, however, that DPC may have other sites of action, because it causes a decrease in V_t when added to unstimulated tubules, which is the reverse of what would be expected from blocking a transcellular Cl^- conductance (Blumenthal, 2001). Tenmo-ADFb and Achdo-KII do not appear to act at the principal cell basal membrane, because V_b is unchanged despite the large decrease in V_t, which suggests they target the apical membrane, causing V_a to decline from ~84 mV to ~55 mV lumen positive.

Does Tenmo-ADFb act at a kinin receptor?

In *T. molitor*, the antidiuretic factor Tenmo-ADFb has been localized immunohistochemically to two pairs of lateral neurosecretory cells located anteriorly in the protocerebrum, axons from which project posteriorly and enter a plexus that appears to be a neurohaemal release site (Eigenheer et al., 2003). Cricket heads have been examined for the presence of Tenmo-ADFb-like immunoreactive material using the same antiserum and methods as those employed in the Eigenheer et al. study (Eigenheer et al., 2003), but without success (L. Schoofs, personal communication). Possibly, *A. domesticus* has an ADFb-like peptide, but it is so dissimilar from the beetle peptide that it is not recognised by the antiserum, which would be consistent with the low potency of Tenmo-ADFb in the cricket diuretic assay. Alternatively, crickets may lack an ADFb-like peptide, in which case the diuretic activity of Tenmo-ADFb could be due to it binding and activating a kinin receptor, which would account for the similar effects of Tenmo-ADFb and Achdo-KII on cricket tubules.

Considerable information is available about the structural requirements for kinin activity in cricket tubules. The minimal sequence requirement for diuretic activity is a C-terminal amidated pentapeptide (Phe-Xxx^1-Xxx^2-Trp-Gly-NH_2; where X^1 is Asn, His, Ser, Tyr or Phe, and X^2 is Ala, Pro or Ser) (Coast et al., 1990). Within this 'active core', residues one (Phe), four (Trp) and five (Gly-NH_2) are invariant, and both Phe and Trp are essential for activity (Roberts et al., 1997). In the active conformation, the two aromatic residues are brought into close proximity on one surface of the molecule, which adopts a type VI β-turn (Nachman et al., 2002). Little is known about the structure–activity relationships of Tenmo-ADFb, but the minimal sequence requirement for diuretic activity in cricket tubules appears to encompass residues 7–12 (Lys-Pro-His-Ile-Tyr-Gly-OH). This sequence has virtually nothing in common with the kinin active core. Importantly, it lacks the Phe and Trp residues that are critical for diuretic activity and is non-amidated, which suggests it is unlikely to interact with a kinin receptor.

In conclusion, Tenmo-ADFb has diuretic rather than antidiuretic activity on cricket tubules. Its effects on ion and fluid transport, and on tubule electrophysiology, are indistinguishable from those of Achdo-KII, although it is considerably less potent. Our data suggest that both peptides stimulate secretion by opening a transepithelial chloride conductance pathway but that they most likely act at different receptors.

We gratefully acknowledge the technical support of Alan Tyler (Birkbeck). This work was supported in part by a NATO Collaborative Research Grant to G.M.C. and R.J.N., and an NIH Grant to D.A.S.

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