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Mosquito natriuretic peptide identified as a calcitonin-like diuretic hormone in *Anopheles gambiae* (Giles)

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Summary

Mosquito natriuretic peptide (MNP), an uncharacterised peptide from the yellow fever mosquito, *Aedes aegypti*, acts *via* cyclic AMP to stimulate secretion of Na^+-rich urine by opening a Na^+ conductance in the basolateral membrane of Malpighian tubule principal cells. Corticotropin releasing factor (CRF)-related peptides and calcitonin (CT)-like diuretic peptides use cyclic AMP as a second messenger and were therefore considered likely candidates for MNP. BLAST searches of the genome of the malaria mosquito *Anopheles gambiae*, gave sequences for the CRF-related peptide Anoga-DH_{44} and the CT-like peptide Anoga-DH_{31}, which were synthesised and tested for effects on Malpighian tubules from *An. gambiae* and *Ae. aegypti*, together with 8-bromo-cyclic AMP.

The cyclic AMP analogue stimulated secretion of Na^+-rich urine by *An. gambiae* Malpighian tubules, reproducing the response to MNP in *Ae. aegypti*. It also depolarised the principal cell basolateral membrane voltage (V_b) while hyperpolarising the transepithelial voltage (V_t) to a similar extent.

Anoga-DH_{44} and Anoga-DH_{31} stimulated production of cyclic AMP, but not cyclic GMP, by Malpighian tubules of *An. gambiae*. Both peptides had diuretic activity, but only Anoga-DH_{31} had natriuretic activity and stimulated fluid secretion to the same extent as 8-bromo-cyclic AMP. Likewise, Anoga-DH_{31} reproduced the effects of cyclic AMP on tubule electrophysiology, whereas Anoga-DH_{44} initially hyperpolarised V_b and depolarised V_t, which is the opposite of the effect of Anoga-DH_{31}.

Anoga-DH_{44} and Anoga-DH_{31} were also tested for effects on fluid secretion and ion transport by *Ae. aegypti* tubules. As in *An. gambiae*, the CRF-related peptide Anoga-DH_{44} had a non-specific effect on the transport of Na^+ and K^+, whereas the CT-like peptide Anoga-DH_{31} specifically stimulated transepithelial Na^+ transport.

We conclude that the CT-like peptide Anoga-DH_{31} is the previously uncharacterised mosquito natriuretic peptide.

Key words: excretion, Malpighian tubule, diuretic hormone, natriuresis, mosquito, *Anopheles gambiae*, *Aedes aegypti.*

Introduction

Female mosquitoes ingest blood meals that are equivalent to more than twice their unfed body mass, and engorged animals can fly only with great difficulty, making them more prone to predation (Roitberg et al., 2003). The blood meal serves primarily as a source of protein for egg development, but it also contains considerable amounts of unwanted salts and water that threaten haemolymph homeostasis. To counter this, a rapid natriuresis and diuresis commences even before the blood meal is completed, and ~40% of the imbibed plasma volume and salt are voided within 1–2 h of feeding (Williams et al., 1983). Indeed, within 20 min of completing the meal, a volume of fluid equal to that of the haemolymph of an unfed insect is absorbed from the midgut and expelled through the anus.

Endocrine control of this post-prandial diuresis has been extensively studied in the yellow fever mosquito, *Aedes aegypti* (Beyenbach, 2003). Between blood meals, the female conserves water and voids little or no urine, but, during the peak phase of diuresis, clear drops of NaCl-rich urine are eliminated from the anus every 12–15 s (Coast et al., 2002; Williams et al., 1983). The stimulus for this diuresis and natriuresis is mosquito natriuretic peptide (MNP), which is released into the haemolymph from structures within the head when the insect takes a blood meal (Beyenbach and Petzel, 1987). MNP acts *via* cyclic AMP to stimulate secretion of Na^+-rich primary urine by the five Malpighian (renal) tubules. Its effects are duplicated by a membrane permeant analogue of cyclic AMP (dibutyryl-cyclic AMP), which has been shown to

depolarise the basolateral membrane voltage (V_b) of Malpighian tubule principal cells and to hyperpolarise the transepithelial voltage (V_t) by a similar amount (Beyenbach, 2003; Sawyer and Beyenbach, 1985). These changes are accompanied by reductions in transepithelial resistance and in the fractional resistance of the basolateral membrane and have been attributed to an increase in the Na^+ conductance of the basolateral membrane (Beyenbach, 2003). In addition, dibutyryl-cyclic AMP activates a bumetanide-sensitive $Na^+/K^+/2Cl^-$ cotransporter (Hegarty et al., 1991). The actions of cyclic AMP make more Na^+ available for transport into the tubule lumen *via* cation/proton antiports in the principal cell apical membrane. This additional Na^+ (along with Cl^- as a counterion) is accompanied by an osmotically equivalent volume of water, and tubule secretion is elevated >7-fold while the Na^+:K^+ concentration ([Na^+]:[K^+]) ratio of the secreted urine increases from unity to approximately 10 (Beyenbach, 2003).

MNP is thought to belong to the corticotropin releasing factor (CRF)-related family of insect diuretic hormones, which use cyclic AMP as their second messenger (Beyenbach, 2003). In support of this, a CRF-related diuretic hormone (Culsa-DH) from the salt-water mosquito *Culex salinarius* stimulates cyclic AMP production by *Ae. aegypti* Malpighian tubules (Cady and Hagedorn, 1999b) and has diuretic and natriuretic activity (Clark et al., 1998b), although the latter effects are small in comparison with those of MNP and exogenous cyclic AMP. Additionally, Culsa-DH causes a biphasic change in the transepithelial voltage of isolated perfused Malpighian tubules (Clark et al., 1998a), which resembles the response to MNP, and stimulates urine production *in vivo* (Cady and Hagedorn, 1999a).

Among the regulatory peptide genes identified in the genome of the malaria mosquito *Anopheles gambiae* (Holt et al., 2002; Riehle et al., 2002) are those encoding orthologues of peptides known to have diuretic activity, namely CRF-related DH, myokinins, calcitonin (CT)-like DH, CAP_{2b} (capa) and tachykinins (Schooley et al., 2005). Of particular interest for the identification of MNP are the CRF-related and CT-like peptides (Anoga-DH_{44} and Anoga-DH_{31}, respectively) since their *Drosophila melanogaster* homologues act *via* cyclic AMP in stimulating Malpighian tubule secretion (Cabrero et al., 2002; Coast et al., 2001). Here, we describe the effects of synthetic Anoga-DH_{44} and Anoga-DH_{31} on fluid and electrolyte (Na^+ and K^+) transport by Malpighian tubules of female *An. gambiae* and *Ae. aegypti*. We show that the CT-like diuretic hormone is the mosquito natriuretic factor described by Petzel et al. (1985), later designated MNP (Beyenbach and Petzel, 1987).

Materials and methods

Mosquitoes

Newly emerged females of the G3 strain of *An. gambiae* (Giles) were obtained from the London School of Hygiene and Tropical Medicine (LSHTM). This strain originated from McCarthy Island in Gambia (West Africa) and was colonised at LSHTM in 1975. Mosquitoes were reared according to methods developed at the LSHTM. The LSHTM also supplied newly emerged females of their AeAe strain of *Aedes aegypti* (L.). Adult mosquitoes were held at 27°C under a 16 h:8 h light:dark regime and fed a 2.5% solution of glucose.

Fluid secretion assay

Malpighian tubules were removed from adult females at 3–10 days post-emergence. Insects were anaesthetised by chilling at 4°C and decapitated prior to removing the Malpighian tubules under *Aedes* saline with the following composition (in mmol l^{-1}): NaCl, 150; $NaHCO_3$, 1.8; KCl, 3.4; $CaCl_2$, 1.7; $MgSO_4$, 4.1; glucose, 5; Hepes (*N*-2-hydroxyethylpiperazine-*N*′2-ethanesulphonic acid), 25; pH adjusted to 7.1 with 1 mol l^{-1} NaOH (Clark et al., 1998b). Tubule secretion assays were performed as described by Clark et al. (1998b) but using 10 µl drops of bathing fluid. A number of different salines were tested for their ability to support fluid secretion (results not shown), and the bathing fluid selected for *An. gambiae* Malpighian tubules was one that comprised a 1:1 mixture of K^+-free *Drosophila* saline (O'Donnell et al., 1996) and Schneider's insect medium (Gibco™; Invitrogen Ltd, Paisley, UK). The *Drosophila* saline had the following composition (in mmol l^{-1}): NaCl, 117.5; $NaHCO_3$, 10.2; NaH_2PO_4, 4.3; $CaCl_2$, 2.0; $MgCl_2$, 8.5; Hepes, 8.6, glucose, 20.0; pH adjusted to 7.0 with NaOH. Test substances were dissolved in the bathing fluid at twice their desired final concentration, and 5 µl were added to the incubation medium, having first removed a 5 µl sample. Similar procedures were used for *Ae. aegypti* Malpighian tubules, but the bathing medium comprised a 1:1 mixture of *Aedes* saline and Schneider's insect medium.

Urine analysis

Electrolyte concentrations (Na^+ and K^+) in urine droplets held beneath water-saturated paraffin oil were measured using ion-selective microelectrodes (Coast et al., 2001; Ianowski and O'Donnell, 2004). Potassium electrodes were based on potassium ionophore I, cocktail A (Fluka, Buchs, Switzerland) and were backfilled with 500 mmol l^{-1} KCl. Sodium electrodes were based on sodium ionophore II, cocktail A (Fluka) and were backfilled with 500 mmol l^{-1} NaCl. For both measurements, the reference electrode was filled with 1 mol l^{-1} LiCl. Potassium electrodes (mean slope 56.1±0.4 mV; N=36) were calibrated in mixed solutions of 200 mmol l^{-1} KCl and NaCl, whereas Na^+ electrodes (mean slope 54.0±0.3 mV; N=26) were calibrated in mixed solutions of 200 mmol l^{-1} NaCl and LiCl. Potassium is known to interfere with Na^+ measurements and this was corrected for as described by Ianowski and O'Donnell (2004).

Electrophysiological measurements

Isolated Malpighian tubules were attached to poly-D-lysine coated cover slips before being transferred to a small (250 µl) chamber that was perfused at 1 ml min^{-1} with *Aedes* saline

containing the following amino acids (in mmol l^{-1}): glycine, 1.7; L-proline, 7.0; L-glutamine, 6.16; L-histidine, 0.95; L-leucine, 0.55; L-lysine, 4.5; L-valine, 1.3. Use of this amino-acid-replete saline, which contains amino acids at the same concentrations as those in the bathing fluid for the fluid secretion assay, resulted in more stable voltages and facilitated the placement of a microelectrode in the tubule lumen. Perfusion was stopped prior to the addition of test compounds and restarted to wash-off. Microelectrodes (120–140 MΩ resistance when filled with 0.1 mol l^{-1} KCl) were fabricated from 1 mm o.d. filament glass tubing (GC100F-75; Clark Electromedical Instruments, Pangbourne, UK) using a microprocessor-controlled vertical pipette puller (World Precision Instruments, Sarasota, FL, USA). They were backfilled with 0.1 mol l^{-1} KCl and connected to a high-impedance electrometer (M-707A; World Precision Instruments) *via* an Ag/AgCl half-cell (World Precision Instruments). An Ag/AgCl reference electrode was placed in a 3 mol l^{-1} KCl reservoir connected to the perfusion chamber *via* a Ringer agar bridge. Basolateral membrane (V_b) and transepithelial voltages (V_t) were measured close to the distal (closed) end of tubules, which were observed using an inverted phase contrast microscope (Nikon TMS, Tokyo, Japan). Large principal cells were selected for recording V_b, and microelectrodes were advanced in steps of 3–7 μm at an oblique angle using a piezoelectric drive (PM-10; World Precision Instruments) until a sudden jump in potential indicated that the basolateral membrane had been impaled. Recordings were deemed successful 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 for 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, Henderson, NV, USA).

Cyclic nucleotide assays

Production of cyclic AMP and cyclic GMP by isolated Malpighian tubules was measured as described by Coast et al. (2001). Malpighian tubules were transferred to Eppendorf tubes (5 or 10 per tube) containing *Drosophila* saline. After incubating for 10 min at room temperature, saline containing 5 mmol l^{-1} 3-isobutyl-1-methylxanthine (IBMX; final concentration 0.5 mmol l^{-1}) was added to each tube. This was followed 10 min later by the addition of IBMX saline alone (controls) or IBMX saline containing either Anoga-DH_{31} or Anoga-DH_{44} (final concentration 1 μmol l^{-1}). The IBMX solution was freshly prepared from a 250 mmol l^{-1} stock solution in DMSO (final DMSO concentration 0.2%). The incubation was terminated after 0–10 min by quenching the tubes in liquid nitrogen. Samples were stored at –80°C until shipped on dry ice to the University of North Wales for the measurement of cyclic AMP and cyclic GMP by radioimmunoassay (RIA; Coast et al., 2001). The cyclic AMP assay was modified by the inclusion of 10 μl crustacean saline (Webster, 1986) in the RIA, which improves the slope of the standard curve (S.G.W., unpublished observation).

Peptide synthesis

The *An. gambiae* CT-like diuretic hormone was located using the Ensembl genome browser with Drome-DH_{31} (Coast et al., 2001) as a query. The 31-residue peptide Anoga-DH_{31} has the predicted sequence TVDFGLSRGYSGAQEAKHR-MAMAVANFAGGP-NH_2 (84% identical to Drome-DH_{31}) and is encoded on a 95 amino acid precursor peptide with accession number XM_321755. The *An. gambiae* CRF-related diuretic hormone, a 44-residue peptide with the predicted sequence TKPSLSIVNPLDVLRQRIILEIARRQMRENTR-QVELNKALLREI-NH_2 (82% identical with Drome-DH_{44}) was also found with Ensembl using Drome-DH_{44} (Cabrero et al., 2002) as a query. There is an intron in the encoding region for Anoga-DH_{44}, located between Glu^{33} and Val^{34}; the gene encoding it is incomplete on both ends, probably due to sequencing errors. Both peptides were synthesized using $N\alpha$-9-fluorenylmethoxycarbonyl (FMOC) chemistry with an Applied Biosystems 431A synthesiser (Foster City, CA, USA). For each synthesis, Rink MBHA amide resin (Novabiochem, San Diego, CA, USA) was used on a 0.1 mmol scale. We activated FMOC–amino acids with 1-hydroxybenzotriazole in 1-methyl-2-pyrrolidinone in the presence of dicyclohexylcarbodiimide for amino acid activation, with a 10-fold molar excess of acylating species. Six 22 min coupling cycles were employed (King, 1996; http://www.abrf.org/ABRFNews/1966/December1996/Long_PepSyn.html); no double couplings were performed. Protecting groups were Arg-[2,2,4,6,7-pentamethylbenzo-(2,3-dihydro)-furansulphonyl], Asn(Trityl), Asp(OtBu), Gln(Trityl), Glu(OtBu), His(Trityl), Lys(Boc), Ser(OtBu), Thr(OtBu) and Tyr(OtBu). The dry resin–peptide was cleaved using Reagent K (King et al., 1990). Crude peptides were precipitated and then washed with methyl-*t*-butyl ether. The dried crude peptides were purified to homogeneity with a ThermoSeparations P4000 liquid chromatograph (San Jose, CA, USA). Crude peptide was first purified by cation exchange on a Polysulfoethyl A column (25×2.1 cm; Poly LC, Columbia, MD, USA) eluted at 10 ml min^{-1} using 20 mmol l^{-1} sodium acetate, pH 4.5, and a gradient from 0 to 1 mol l^{-1} NaCl. This removed Arg deletion peptides, which are difficult to separate by reversed phase. Purified fractions were re-purified with a YMC 25×2.0 cm C_8 column (Waters Co., Milford, MA, USA) eluted at 10 ml min^{-1} with an ethanol–water–0.1% trifluoroacetic acid gradient.

Chemicals

Muscakinin (Musdo-K) was a generous gift from Dr R. J. Nachman (USDA, College Station, TX, USA). Unless otherwise stated, all other chemicals were obtained from Sigma-Aldrich.

Statistics

Tests for significance were performed using GraphPad Instat v.3.06 (GraphPad Software, San Diego, CA, USA), with $P<0.05$ being accepted as significant. Dose–response curves with variable slope were fitted using Prism™ v.4.02 (GraphPad Software).

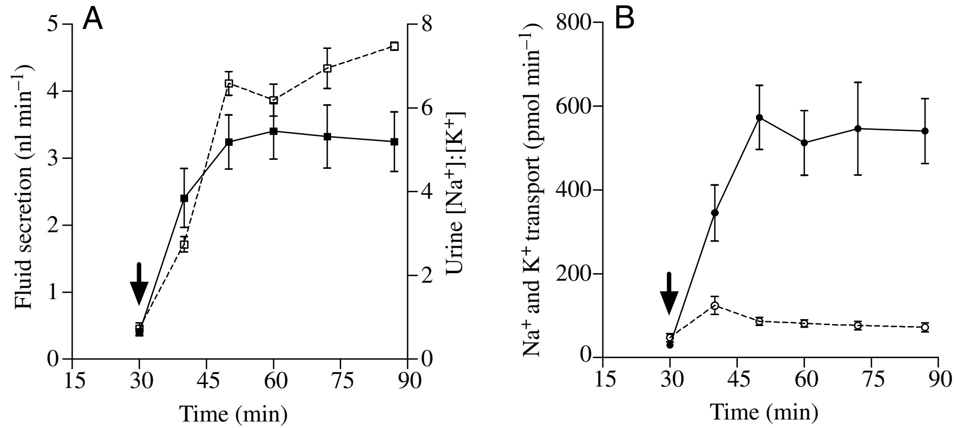

Fig. 1. Exogenous 8-bromo-cyclic AMP stimulates secretion of Na^+-rich urine by *An. gambiae* Malpighian tubules. Fluid secretion and urine Na^+ and K^+ concentrations were initially measured over a 30 min control period and then at 10–15 min intervals after the addition of 1 mmol l^{-1} 8-bromo-cyclic AMP. Data points show the means ± S.E.M. for five tubules. (A) Fluid secretion (solid line) and the tubule fluid $[Na^+]$:$[K^+]$ ratio (broken line) increase after the addition of 8-bromo-cyclic AMP. (B) This reflects the selective stimulation of transepithelial Na^+ transport (solid line) compared with K^+ transport (broken line). Arrows show the time of addition of 8-bromo-cyclic AMP.

Results

Effect of exogenous cyclic AMP

Prior to examining the effects of Anoga-DH_{31} and Anoga-DH_{44} on *An. gambiae* Malpighian tubules, we determined whether exogenous cyclic AMP had effects similar to those reported in *Ae. aegypti*. Fluid secretion and tubule fluid Na^+ and K^+ concentrations were measured over 30 min under control (unstimulated) conditions and then at 10–15 min intervals in the presence of a membrane permeant cyclic AMP analogue (8-bromo-cyclic AMP; 1 mmol l^{-1}). The cyclic AMP analogue stimulated Malpighian tubule secretion 10-fold, and the $[Na^+]$:$[K^+]$ ratio of the primary urine increased from 0.7 to 7.5 (Fig. 1A). Transepithelial Na^+ transport (the product of ion concentration and rate of secretion) increased >18-fold compared with only a 2.6-fold increase for K^+, which declined after 10 min (Fig. 1B). The effect of 8-bromo-cyclic AMP on ion and fluid transport by *An. gambiae* Malpighian tubules thus reproduced the diuretic and natriuretic activities of the membrane permeant dibutyryl analogue of cyclic AMP in *Ae. aegypti*.

Likewise, the effects of 8-bromo-cyclic AMP on Malpighian tubule electrophysiology in *An. gambiae* were similar to those reported for dibutyryl-cyclic AMP in *Ae. aegypti*. Absolute values for V_t (33.7±3.7 mV; N=28) and principal cell V_b (–68.5±1.7 mV; N=54) varied considerably between tubules under control conditions. The addition of 100 µmol l^{-1} 8-bromo-cyclic AMP resulted in an immediate depolarisation of V_b by 55.8±5.3 mV (N=9) while V_t hyperpolarised by 39.1±10.8 mV (N=5; Fig. 2). These values are not significantly different (P=0.142; unpaired *t*-test), which suggests that the apical membrane voltage (V_a=V_t–V_b) remains relatively constant. However, given the variability of V_t and V_b, which were measured in different Malpighian tubules, a small change in V_a might have gone undetected.

Effects of Anoga-DH_{44} and Anoga-DH_{31} on ion and fluid transport

Fluid secretion and tubule fluid Na^+ and K^+ concentrations were measured over 30 min under control conditions and then in the presence of 1 µmol l^{-1} of either Anoga-DH_{44} or Anoga-DH_{31}. After 60 min, all tubules were challenged with 1 mmol l^{-1} 8-bromo-cyclic AMP to establish maximum rates of ion and fluid transport. Tubule secretion was increased 3-fold by Anoga-DH_{44} (Fig. 3A) but then more than doubled after the addition of 8-bromo-cyclic AMP. Importantly, the $[Na^+]$:$[K^+]$ ratio of the secreted urine fell from 0.70±0.09 to 0.45±0.04 (N=5; P<0.05) in response to Anoga-DH_{44} but increased to 2.98±0.30 after the addition of 8-bromo-cyclic AMP (Fig. 3A). This reflects the selective stimulation of transepithelial Na^+ transport by 8-bromo-cyclic AMP, but not by Anoga-DH_{44} (Fig. 3B).

Fig. 4 shows the results of the same experiment with Anoga-DH_{31}. Fluid secretion was increased 10-fold by Anoga-DH_{31}

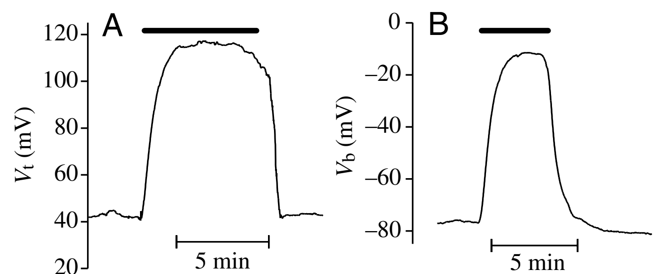

Fig. 2. Representative recordings of (A) transepithelial voltage (V_t) and (B) principal cell basolateral membrane voltage (V_b) in Malpighian tubules challenged with 100 µmol l^{-1} 8-bromo-cyclic AMP. Exogenous cyclic AMP hyperpolarises V_t and depolarises V_b to a similar extent. Horizontal bars indicate when 8-bromo-cyclic AMP was present in the bath.

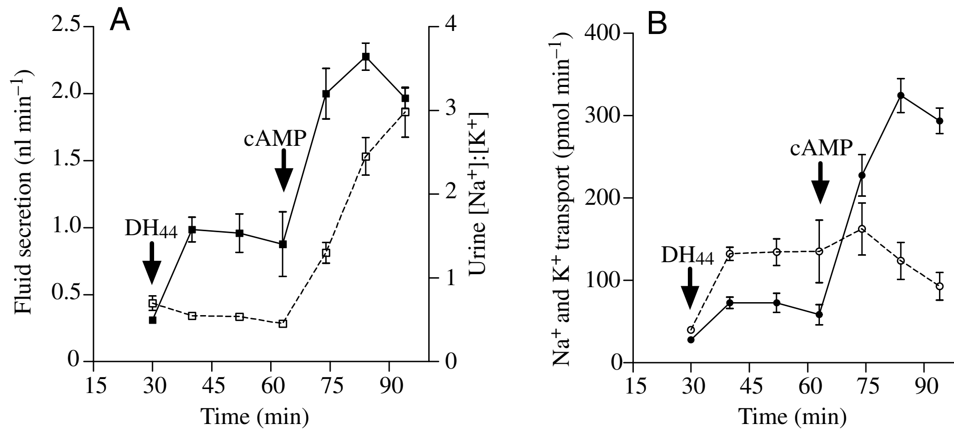

Fig. 3. Anoga-DH$_{44}$ has diuretic activity but does not selectively stimulate Na^+ transport. Fluid secretion and urine Na^+ and K^+ concentrations were initially measured over a 30 min control period and then at 10–15 min intervals after the addition of 1 μmol l^{-1} Anoga-DH$_{44}$ alone (first arrow) and then in combination with 1 mmol l^{-1} 8-bromo-cyclic AMP (second arrow). Data points show the means ± S.E.M. for five tubules. (A) Fluid secretion (solid line) is increased 3-fold by Anoga-DH$_{44}$, whereas the urine [Na^+]:[K^+] ratio (broken line) fell slightly. The addition of 8-bromo-cyclic AMP to the same batch of tubules further accelerates fluid secretion and increases the [Na^+]:[K^+] ratio. (B) Both Na^+ (solid line) and K^+ (broken line) transport are increased by Anoga-DH$_{44}$, whereas 8-bromo-cyclic AMP selectively stimulates the secretion of Na^+.

and was not stimulated further by the addition of 8-bromo-cyclic AMP (Fig. 4A). The response to Anoga-DH$_{31}$ was associated with a marked increase in the [Na^+]:[K^+] ratio of the secreted urine, from 0.64±0.08 (N=5) to 5.91±1.69. The ratio increased to 6.30±1.08 in the presence of 8-bromo-cyclic AMP, but this was not significantly different (P=0.604; paired t-test) from that obtained with Anoga-DH$_{31}$ alone. Thus, in marked contrast to Anoga-DH$_{44}$, the CT-like diuretic hormone selectively stimulated transepithelial Na^+ transport, which was increased 16-fold compared with only a 2.4-fold increase for K^+ (Fig. 4B).

To demonstrate further the different effects of the two diuretic hormones on ion and fluid transport, a group of Malpighian tubules were challenged over 30 min intervals with first Anoga-DH$_{44}$ alone and then together with Anoga-DH$_{31}$; the final concentration of both peptides was 1 μmol l^{-1}. Under control conditions, tubule secretion was 0.36±0.01 nl min^{-1} (N=5) and the [Na^+]:[K^+] ratio of the secreted urine was 1.17±0.36. Anoga-DH$_{44}$ produced a 3-fold increase in fluid secretion but had no effect on the urine [Na^+]:[K^+] ratio, whereas the ratio increased >7-fold after the addition of Anoga-DH$_{31}$ to tubules already exposed to Anoga-DH$_{44}$, and there was a further 2-fold increase in the rate of secretion (Fig. 5). The change in [Na^+]:[K^+] ratio reflects the markedly different effects of the two peptides on transepithelial Na^+ and K^+ transport. In the presence of Anoga-DH$_{44}$, Na^+ transport increased from 35±4 pmol min^{-1} to a maximum of 109±17 pmol min^{-1} but reached 399±55.2 pmol min^{-1} after the

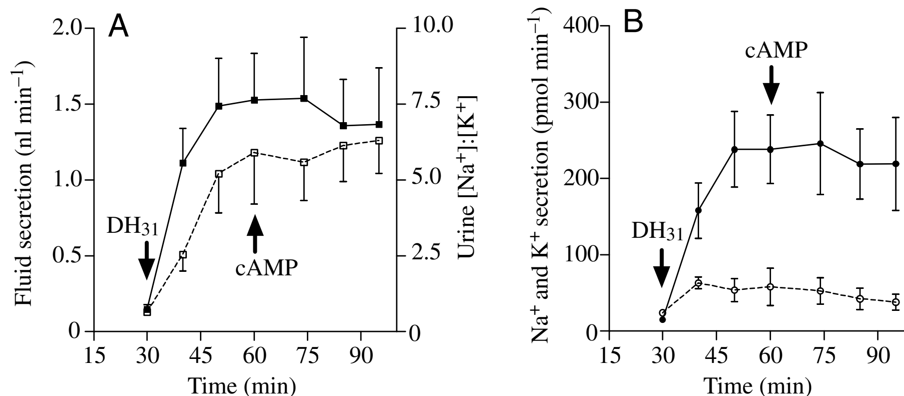

Fig. 4. Anoga-DH$_{31}$ stimulates diuresis and natriuresis, mimicking the actions of exogenous cAMP. Fluid secretion and urine Na^+ and K^+ concentrations were initially measured over a 30 min control period and then at 10–15 min intervals after the addition of 1 μmol l^{-1} Anoga-DH$_{31}$ alone (first arrow) and then in combination with 1 mmol l^{-1} 8-bromo-cyclic AMP (second arrow). Data points show the means ± S.E.M. for five tubules. (A) Anoga-DH$_{31}$ stimulates fluid secretion (solid line) and increases the urine [Na^+]:[K^+] ratio (broken line) to the same extent as 8-bromo-cyclic AMP. (B) Transepithelial Na^+ transport (solid line) is selectively stimulated by Anoga-DH$_{31}$, which has relatively little effect on K^+ transport (broken line).

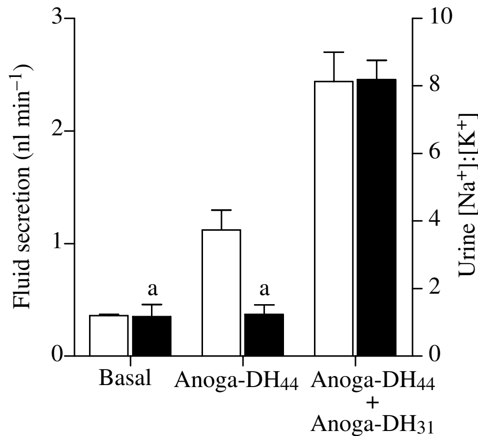

Fig. 5. Sequential addition of Anoga-DH_{44} and Anoga-DH_{31} to the same batch of tubules demonstrates that only the latter has pronounced natriuretic activity. Fluid secretion and urine Na^+ and K^+ concentrations were measured over 30 min intervals under control conditions and after the addition of 1 µmol l^{-1} Anoga-DH_{44} alone and in combination with 1 µmol l^{-1} Anoga-DH_{31}. Bars represent the means + S.E.M. for five tubules. Anoga-DH_{44} stimulates fluid secretion (open bars) without affecting the [Na^+]:[K^+] ratio (solid bars) of the secreted urine, which increases dramatically after the addition of Anoga-DH_{31} along with a further acceleration of urine flow. Identical letters indicate values that do not differ significantly (P>0.05).

addition of Anoga-DH_{31}. On the other hand, K^+ transport was increased from 36±7 pmol min^{-1} to 105±29 pmol min^{-1} by Anoga-DH_{44} but then fell to 47±7 pmol min^{-1} following the addition of Anoga-DH_{31}.

Continuous recording of tubule fluid K^+ concentration

Fig. 6 shows real-time recordings of the K^+ concentration in urine secreted by tubules challenged with either Anoga-DH_{44} or Anoga-DH_{31}. The Na^+ concentration is calculated on the basis of the sum of [Na^+] and [K^+] being virtually constant at 200 mmol l^{-1}. Following a 30 min equilibration period, a drop of secreted fluid was removed, and a K^+ selective microelectrode, together with a reference electrode, was positioned in the newly secreted urine. After 2–3 min, time to establish the K^+ concentration of the urine under control conditions, tubules were challenged with either Anoga-DH_{44} or Anoga-DH_{31} (1 µmol l^{-1} final concentration). The representative recording in Fig. 6A shows the effect of the CRF-related peptide Anoga-DH_{44}. The K^+ concentration of the secreted urine was initially 75 mmol l^{-1} but fell to 65 mmol l^{-1} within 10 min of adding the CRF-related diuretic hormone. This reflects a small increase in Na^+ transport relative to K^+, but this was not sustained and the K^+ concentration was constant over the next 20 min. Fig. 6B shows similar data for a tubule stimulated with Anoga-DH_{31}. The K^+ concentration of the secreted urine fell exponentially from about 102 mmol l^{-1} to 30 mmol l^{-1} over 30 min. The reciprocal increase in the Na^+ concentration of the secreted urine reflects the natriuretic activity of the calcitonin-like diuretic hormone.

Effects of Anoga-DH_{44} and Anoga-DH_{31} on tubule electrophysiology

Representative recordings of the effects of 100 nmol l^{-1} Anoga-DH_{44} and 100 nmol l^{-1} Anoga-DH_{31} on the basolateral membrane voltage of Malpighian tubule principal cells are shown in Fig. 7. Addition of Anoga-DH_{44} resulted in a triphasic change in membrane voltage (Fig. 7A), which initially hyperpolarised by 6.1±1.3 mV (N=15; Phase 1) and then depolarised by 35.4±4.7 mV (Phase 2) before returning to close to its control value in the continued presence of the peptide (Phase 3). The transepithelial voltage changed in a similar manner (data not shown), first depolarising by 13.5±4.1 mV (N=8) and then hyperpolarising by 28.8±6.5 mV before returning to close to the control value. The magnitude of the changes in transepithelial voltage was not significantly different from that in the basolateral membrane voltage during both Phase 1 (P=0.124, unpaired t-test with Welch correction) and Phase 2 (P=0.418, unpaired t-test) of the response, which suggests that the apical membrane voltage is not affected by Anoga-DH_{44}.

The effect of Anoga-DH_{31} on the voltage across the basolateral membrane was very different in that the initial hyperpolarising phase (Phase 1) was absent (Fig. 7B). Instead, there was an immediate depolarisation of 41.9±2.5 mV (N=30) followed by a variable degree of repolarisation, which left the

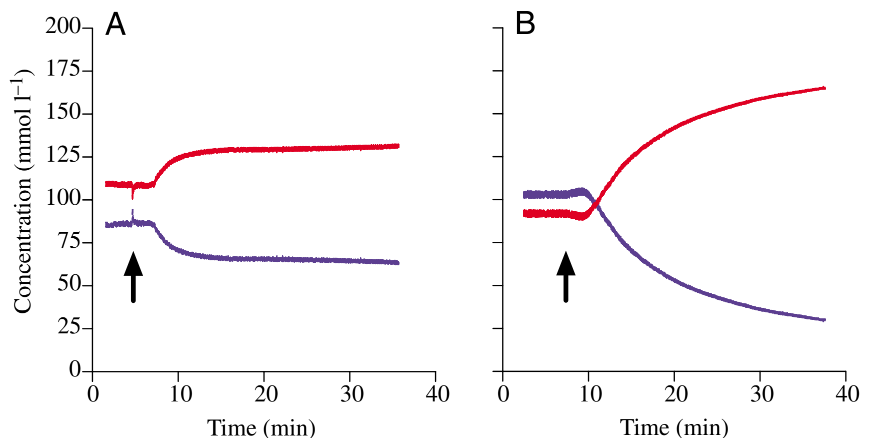

Fig. 6. Representative recordings of the K^+ concentration (blue line) of urine secreted by tubules challenged with either (A) 1 µmol l^{-1} Anoga-DH_{44} or (B) 1 µmol l^{-1} Anoga-DH_{31}. The concentration of Na^+ (red line) in the secreted fluid was calculated assuming the sum of Na^+ and K^+ concentrations was 200 mmol l^{-1}. Arrows show the time of addition of the diuretic peptides.

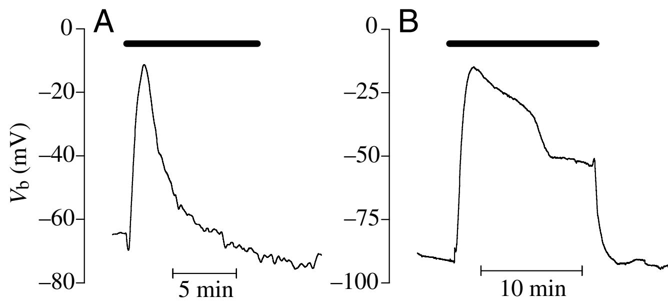

Fig. 7. Representative recordings of principal cell basolateral membrane voltage (V_b) in tubules stimulated with either (A) 100 nmol l^{-1} Anoga-DH_{44} or (B) 100 nmol l^{-1} Anoga-DH_{31}. The response to Anoga-DH_{44} is triphasic, commencing with a transient hyperpolarisation (Phase 1) followed by the depolarisation (Phase 2) and repolarisation (Phase 3) of V_b before the peptide is washed off. Horizontal bars indicate when the peptides were present in the bath.

membrane depolarised relative to its control value until the peptide was washed off. Similar changes were recorded in the transepithelial voltage (data not shown), which hyperpolarised by 37.2±4.9 mV (N=14) before dropping back to a plateau that was higher than during the control period. The magnitude of the initial change in basolateral and transepithelial voltages did not differ significantly (P=0.347), and the voltage across the apical membrane is therefore likely to be unaffected.

Differences between the actions of Anoga-DH_{44} and Anoga-DH_{31} on tubule electrophysiology are highlighted in Fig. 8, which shows the basolateral membrane voltage recorded in a single principal cell challenged sequentially with 100 nmol l^{-1} of each of the peptides and then with 100 μmol l^{-1} 8-bromo-cyclic AMP. The triphasic response to Anoga-DH_{44} is readily distinguishable by the brief hyperpolarising phase that precedes the depolarisation and by the membrane potential repolarising before the peptide is washed off. The initial hyperpolarisation is absent from the responses to Anoga-DH_{31}

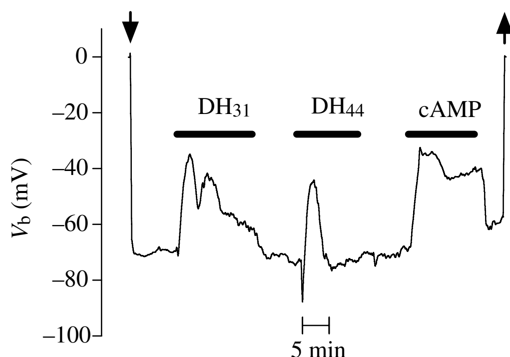

Fig. 8. Representative recording of the basolateral membrane voltage (V_b) in a principal cell challenged sequentially with 100 nmol l^{-1} Anoga-DH_{31}, 100 nmol l^{-1} Anoga-DH_{44} and 100 μmol l^{-1} 8-bromo-cyclic AMP. Note that only Anoga-DH_{44} gives a triphasic response beginning with a brief hyperpolarisation. Horizontal bars indicate when peptides or cyclic AMP analogue were present in the bath. Arrows show when the principal cell was impaled (downward arrow) and the microelectrode withdrawn (upward arrow).

and cyclic AMP, and the membrane remains depolarised until the secretagogues are washed off.

Dose–response relations for Anoga-DH_{31}

The diuretic activity of Anoga-DH_{31} was investigated on *An. gambiae* tubules at concentrations ranging from 1 nmol l^{-1} to 1000 nmol l^{-1}. Fluid secretion was measured over 45 min under control conditions and then for 30 min in the presence of Anoga-DH_{31}. Finally, 1 mmol l^{-1} 8-bromo-cyclic AMP was added to all tubules and fluid secretion measured over a further 30 min. Diuretic activity was calculated as the difference between rates of fluid secretion in the control and experimental periods. The length of tubule within the bathing fluid drop varied considerably and the diuretic activity of Anoga-DH_{31} was therefore normalised by expressing it as a percentage of the diuretic activity of 1 mmol l^{-1} 8-bromo-cyclic AMP.

Fluid secretion was significantly stimulated (P<0.05; paired t-test) by concentrations of Anoga-DH_{31} of ⩾10 nmol l^{-1}, and the response at 1000 nmol l^{-1} did not differ from that obtained with 1 mmol l^{-1} 8-bromo-cyclic AMP (P=0.564). The apparent EC_{50} was 50 nmol l^{-1} with 95% confidence limits of 41–59 nmol l^{-1}.

Cyclic nucleotide production by isolated tubules challenged with Anoga-DH_{44} and Anoga-DH_{31}

The production of cyclic AMP was measured over 10 min by Malpighian tubules (five per tube) isolated from *An. gambiae* and incubated in *Drosophila* saline containing 500 μmol l^{-1} IBMX. Cyclic AMP levels in the saline controls were below the level of detection in the RIA, which was 15 fmol $tube^{-1}$. However, significant (P<0.01) amounts of cyclic AMP were detected in the presence of 1 μmol l^{-1} Anoga-DH_{31}, reaching the equivalent of 32.7±7.3 fmol $tubule^{-1}$ (N=8). Cyclic AMP levels in tubules challenged with 1 μmol l^{-1} Anoga-DH_{44} were more variable (12.7±7.5 fmol $tubule^{-1}$; N=8) and not significantly different from zero (P=0.134). The experiment was therefore repeated with 10 Malpighian tubules per assay tube and incubating for 0, 2, 5 and 10 min in saline containing 500 μmol l^{-1} IBMX with or without the addition of 1 μmol l^{-1} Anoga-DH_{44}. After incubating for 2 min in the presence of Anoga-DH_{44}, cyclic AMP levels had increased significantly (P<0.001; Mann–Whitney test) from 7.0±1.7 fmol $tubule^{-1}$ (N=10) to 54.2±12.7 fmol $tubule^{-1}$ (N=5). Cyclic AMP levels were not increased further by incubating for 5 min (32.6±7.5 fmol $tubule^{-1}$; N=5) and 10 min (40.7±7.8 fmol $tubule^{-1}$; N=5) but were significantly higher (P<0.01; Mann–Whitney test) than the corresponding controls, which were 2.2±2.2 fmol $tubule^{-1}$ (N=5) and 5.4±1.1 fmol $tubule^{-1}$ (N=5), respectively.

Following a 10 min incubation in IBMX saline containing either 1 μmol l^{-1} Anoga-DH_{44} or 1 μmol l^{-1} Anoga-DH_{31}, cyclic GMP levels were equivalent to 0.3±0.1 fmol $tubule^{-1}$ (N=4) and 0.3±0.1 fmol $tubule^{-1}$ (N=5), respectively, which were not significantly different (P=0.965) from the saline controls (0.3±0.1 fmol $tubule^{-1}$; N=6).

Table 1. *Effect of Anoga-DH$_{44}$ and Anoga-DH$_{31}$ on fluid secretion and ion (Na^+ and K^+) transport by Malpighian tubules of the yellow fever mosquito (*Ae. aegypti*)*

	Fluid secretion (nl min^{-1})	[Na$^+$]:[K$^+$]	K$^+$ transport (pmol min^{-1})	Na$^+$ transport (pmol min^{-1})
*Anoga-DH$_{44}$ and 8-bromo-cyclic AMP (*N=6*)*				
Basal	0.29±0.10	0.89±0.21^{a}	35.4±15.8^{b}	16.6±4.1
Anoga-DH$_{44}$ (1 µmol l^{-1})	0.78±0.17	0.96±0.25^{a}	74.4±20.7^{b,c}	50.9±11.1
+ cAMP (1 mmol l^{-1})	2.99±0.47	3.40±0.63	137.6±35.7^{c}	383.8±64.7
*Anoga-DH$_{31}$ and 8-bromo-cyclic AMP (*N=6*)*				
Basal	0.56±0.10	0.96±0.18	57.5±10.2^{e}	48.1±9.2
Anoga-DH$_{31}$ (1 µmol l^{-1})	3.57±0.51^{d}	4.33±0.34	123.9±22.3^{f}	523.1±75.0^{g}
+ cAMP (1 mmol l^{-1})	3.28±0.38^{d}	6.22±0.41	82.9±14.2^{e,f}	495.7±59.3^{g}
*Anoga-DH$_{44}$ and Anoga-DH$_{31}$ (*N=8*)*				
Basal	0.28±0.06	0.45±0.04^{h}	38.1±8.9	16.2±2.8
Anoga-DH$_{44}$ (1 µmol l^{-1})	0.73±0.13	0.39±0.03^{h}	99.4±18.2^{i}	38.4±8.2
Anoga-DH$_{31}$ (1 µmol l^{-1})	2.09±0.25	2.27±0.20	120.0±12.8^{i}	259.1±35.6

Values are means ± S.E.M. for the number of determinations shown in parentheses. Identical letters indicate values that do not differ significantly (P>0.05).

Effect of Anoga-DH$_{44}$ and Anoga-DH$_{31}$ on ion and fluid transport by Ae. aegypti *tubules*

Anoga-DH$_{31}$ has both diuretic and natriuretic activity in *An. gambiae*, and this CT-like peptide is the most likely candidate for the MNP described in *Ae. aegypti* by Petzel et al. (1985). We sought to confirm this by testing both peptides on Malpighian tubules from the yellow fever mosquito. Secretion was measured over 30 min under control conditions and for 30 min periods in the presence of either Anoga-DH$_{44}$ or Anoga-DH$_{31}$ (final concentration 1 µmol l^{-1}) alone and then in combination with 1 mmol l^{-1} 8-bromo-cyclic AMP. The results were very similar to those obtained with *An. gambiae* and are summarised in Table 1. Anoga-DH$_{44}$ stimulated fluid secretion but had no effect on the [Na$^+$]:[K$^+$] ratio of the secreted urine. Both parameters were increased >3-fold after the addition of 8-bromo-cyclic AMP, reflecting a dramatic rise in transepithelial Na$^+$ transport, whereas K$^+$ transport was unchanged. In marked contrast, the CT-like peptide Anoga-DH$_{31}$ stimulated fluid secretion and transepithelial Na$^+$ transport to the same extent as exogenous cyclic AMP. To further illustrate the differing effects of the two peptides, one batch of tubules was challenged with 1 µmol l^{-1} Anoga-DH$_{44}$ alone and then in combination with 1 µmol l^{-1} Anoga-DH$_{31}$ (Table 1). As shown previously, Anoga-DH$_{44}$ increased fluid secretion but had an insignificant effect on the urine [Na$^+$]:[K$^+$] ratio. Fluid secretion was further accelerated by the addition of Anoga-DH$_{31}$ and this was associated with a marked increase in the [Na$^+$]:[K$^+$] ratio of the secreted fluid, which reflected the specific stimulation of transepithelial Na$^+$ transport.

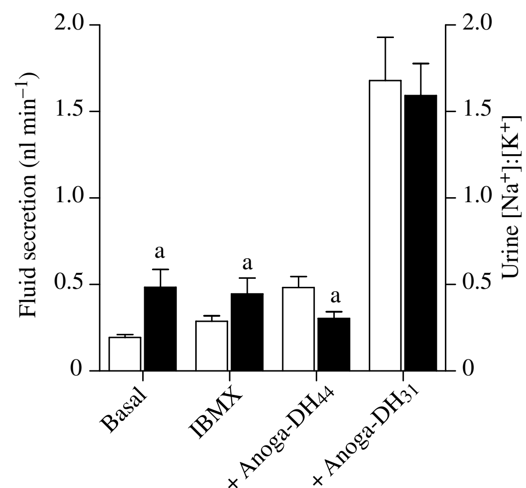

Fig. 9. Anoga-DH$_{44}$ does not selectively stimulate Na$^+$ transport after phosphodiesterase activity is inhibited by IBMX. Fluid secretion and urine Na$^+$ and K$^+$ concentrations were first measured under control conditions (30 min) and then in the presence of 100 µmol l^{-1} IBMX (50 min). Subsequently, the tubules were challenged with 1 µmol l^{-1} Anoga-DH$_{44}$ alone (40 min) and then in combination with 1 µmol l^{-1} Anoga-DH$_{31}$ (40 min) in the continued presence of IBMX. Fluid secretion and ion concentrations were measured at the end of each collection period. Bars represent the means + S.E.M. for eight tubules. Fluid secretion (open bars) increases in the presence of IBMX, but the urine [Na$^+$]:[K$^+$] ratio (solid bars) is unchanged. Anoga-DH$_{44}$ promotes a further increase in secretion but has no effect on the [Na$^+$]:[K$^+$] ratio, whereas both parameters increase dramatically after the addition of Anoga-DH$_{31}$. Identical letters indicate values that do not differ significantly (P>0.05).

Effect of IBMX on the response to Anoga-DH$_{44}$

We have shown that both Anoga-DH$_{44}$ and Anoga-DH$_{31}$ stimulate cyclic AMP production by Malpighian tubules isolated from *An. gambiae* but that only the CT-like peptide has pronounced natriuretic activity. Conceivably, this might be because Anoga-DH$_{31}$ is more effective than Anoga-DH$_{44}$ in elevating intracellular levels of cyclic AMP. To investigate

this, the peptides were tested on Malpighian tubules after cyclic AMP phosphodiesterase activity had been inhibited with IBMX in order to elevate intracellular levels of the second messenger. The results are shown in Fig. 9. Tubule secretion rose during the 50 min incubation with 100 μmol l^{-1} IBMX, while the [Na^+]:[K^+] ratio of the secreted urine was unchanged. The addition of 1 μmol l^{-1} Anoga-DH_{44} to the IBMX saline resulted in a further increase in tubule secretion but had no significant effect (P>0.05; Tukey–Kramer Multiple Comparisons Test) on the [Na^+]:[K^+] ratio. However, with the inclusion of Anoga-DH_{31} (final concentration 1 μmol l^{-1}) in the bathing fluid, the urine [Na^+]:[K^+] ratio increased dramatically, which, taken together with the further acceleration of tubule secretion, represented a 9-fold increase in Na^+ transport (from 17.1±2.1 pmol min^{-1} to 180.2±31.1 pmol min^{-1}) compared with only a 2-fold increase in K^+ transport (from 64.5±8.7 pmol min^{-1} to 114.2±15.0 pmol min^{-1}).

Effect of Musdo-K on the activity of Anoga-DH₃₁

The CRF-related peptides Anoga-DH_{44} and Culsa-DH initially depolarise the transepithelial voltage of Malpighian tubules from *An. gambiae* (present study) and *Ae. aegypti* (Clark et al., 1998a), respectively. Clark et al. (1998a) attribute this initial depolarisation to the Ca^{2+}-dependent opening of a paracellular Cl^- conductance, which characterises the response to the kinin family of insect diuretic peptides and results in a non-selective increase in Na^+ and K^+ transport (Beyenbach, 1995). Conceivably, this non-selective response to activation of the Cl^- conductance pathway could outweigh a cyclic AMP-dependent stimulation of Na^+ transport and thus account for the different effects of Anoga-DH_{31} and Anoga-DH_{44} on cation transport. To test this hypothesis, we determined what effect activating the Cl^- conductance pathway using a diuretic/myotropic kinin had on the response to Anoga-DH_{31}. Since none of the *An. gambiae* kinins were available to us, we used the housefly (*Musca domestica*) kinin, Musdo-K.

Fig. 10 shows a recording of the basolateral membrane voltage from an *An. gambiae* principal cell challenged separately with first 100 nmol l^{-1} Anoga-DH_{31}, then with 100 nmol l^{-1} Musdo-K and finally with a combination of the two peptides. The basolateral membrane was depolarised by Anoga-DH_{31} and hyperpolarised by Musdo-K, which is consistent with the effect of kinin stimulation in *Ae. aegypti* Malpighian tubules (Pannabecker et al., 1993). A combination of the two peptides produced a triphasic response similar to that obtained with Anoga-DH_{44} (cf. Fig. 7A), although the initial hyperpolarisation was more pronounced.

Given that the electrophysiological signature of Anoga-DH_{44} was similar to that obtained with a combination of Anoga-DH_{31} and Musdo-K, we investigated how these two peptides influenced fluid secretion and ion transport. Musdo-K alone (1 μmol l^{-1} final concentration) produced a 4-fold increase in fluid secretion but had no significant effect on the urine [Na^+]:[K^+] ratio (Fig. 11). By contrast, the [Na^+]:[K^+] ratio was increased >6-fold when Anoga-DH_{31} (final concentration 1 μmol l^{-1}) was also added to the same group of tubules. This was accompanied by a 6-fold stimulation of fluid secretion and therefore represented an 18-fold increase in Na^+ transport, from 38±5 pmol min^{-1} (N=8) to 677±93 pmol min^{-1}, compared with a 3-fold increase in K^+ transport, from 128±20 pmol min^{-1} to 378±58 pmol min^{-1}.

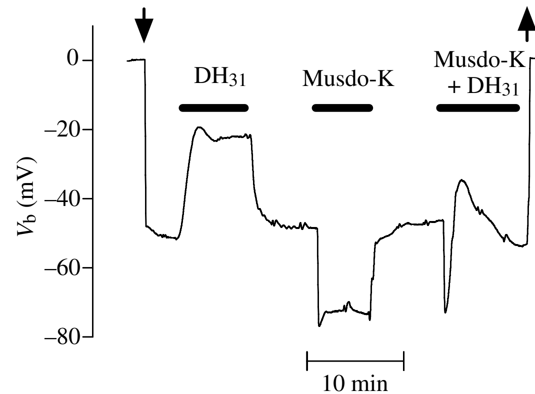

Fig. 10. Representative recording of the basolateral membrane voltage (V_b) in a principal cell challenged separately and together with 100 nmol l^{-1} Anoga-DH_{31} and 100 nmol l^{-1} Musdo-K. Anoga-DH_{31} depolarises and Musdo-K hyperpolarises V_b, but in combination they produce a triphasic response mimicking that obtained with Anoga-DH_{44} (cf. Fig. 7A). Horizontal bars indicate when the peptides were present in the bath. Arrows show when the principal cell was impaled (downward arrow) and the microelectrode withdrawn (upward arrow).

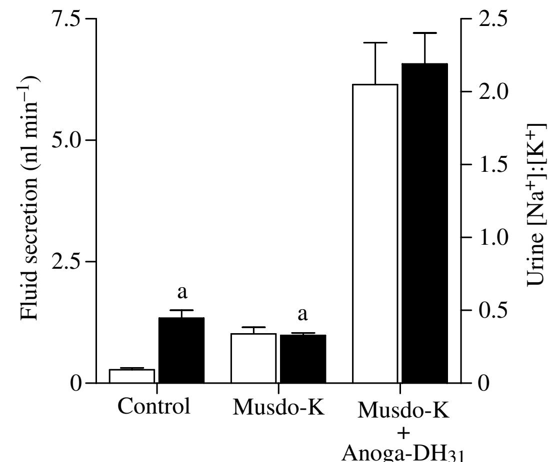

Fig. 11. Anoga-DH_{31} has natriuretic activity in tubules previously challenged with Musdo-K. Fluid secretion and urine Na^+ and K^+ concentrations were measured over 30 min intervals under control conditions and after the addition of 1 μmol l^{-1} Musdo-K alone and then in combination with 1 μmol l^{-1} Anoga-DH_{31}. Bars represent the means + S.E.M. for eight tubules. Fluid secretion (open bars) is stimulated 4-fold by Musdo-K, but the urine [Na^+]:[K^+] ratio (solid bars) is unchanged. Both parameters are increased >6-fold after the addition of Anoga-DH_{31}. Identical letters indicate values that do not differ significantly (P>0.05).

Discussion

The natriuretic peptide of mosquitoes is a CT-like peptide

The mosquito natriuretic peptide identified in an *Ae. aegypti* head extract by Petzel et al. (1985) acts *via* cyclic AMP to stimulate secretion of Na^+-rich urine by Malpighian tubules (Petzel et al., 1987). CRF-related diuretic hormones are known to use cyclic AMP as a second messenger (Coast and Kay, 1994), and Beyenbach (2003) recently suggested that MNP belongs to this peptide family, based upon results from a cross-species study using Culsa-DH. In the present paper, we report the actions of Anoga-DH_{44}, the CRF-related peptide in the malaria mosquito, on Malpighian tubules from *An. gambiae* and *Ae. aegypti*. We show that it has diuretic activity but does not selectively stimulate Na^+ transport, which is the defining feature of MNP. On the other hand, Anoga-DH_{31}, a CT-like peptide that is common to *An. gambiae* and *Ae. aegypti*, has both diuretic and natriuretic activities, stimulating fluid secretion and transepithelial Na^+ transport to the same extent as exogenous 8-bromo-cyclic AMP. We therefore propose that the previously uncharacterised MNP belongs to the CT-like family of insect diuretic hormones.

Effects of Anoga-DH44 and Anoga-DH31 on tubule electrophysiology

Anoga-DH_{44} and Anoga-DH_{31} produce characteristic changes in transepithelial voltage, which are due largely to voltage changes at the principal cell basolateral membrane. The response to the CRF-related peptide is very similar to that reported previously for MNP (fraction III in Petzel et al., 1985) and Culsa-DH (fig. 2 in Clark et al., 1998a), in that the transepithelial voltage briefly depolarises before hyperpolarising. Interestingly, this transient is not seen with either Anoga-DH_{31} or membrane-permeant analogues of cyclic AMP (present study; Sawyer and Beyenbach, 1985) and yet they, rather than Anoga-DH_{44}, mimic the natriuretic activity of MNP. Clark et al. (1998b) attribute the depolarising and hyperpolarising phases of the response to Culsa-DH as being due to the Ca^{2+}-dependent opening of a paracellular Cl^- conductance and the cyclic AMP-mediated stimulation of active cation transport, respectively. Our findings are consistent with this interpretation in that a combination of Anoga-DH_{31} and Musdo-K, a member of the kinin family of diuretic peptides that use Ca^{2+} as a second messenger in activating a Cl^- conductance pathway (O'Donnell et al., 1998; Yu and Beyenbach, 2002), mimic the electrophysiological signature of Anoga-DH_{44}. One way of accounting for the biphasic effect of MNP on transepithelial voltage is to suggest that fraction III (which was dubbed MNP by Petzel et al., 1985) contained Anoga-DH_{31} together with a kinin. In support of this, gel filtration chromatography of fraction III indicated a molecular mass of 1862 Da (Petzel et al., 1986), which is more similar to Aedae-K-I (1593 Da) than to either CT-like or CRF-related diuretic hormones.

Anoga-DH31 and Anoga-DH44 use cyclic AMP as a second messenger but have different effects on ion transport

A paradox to arise from the present study is that, although Anoga-DH_{31} and Anoga-DH_{44} both stimulate cyclic AMP production by isolated Malpighian tubules, only the former has natriuretic activity. As yet, we cannot explain this, but we are able to rule out a number of possibilities. First, the peptides could be acting on functionally different populations of principal cells, only one of which responds to a rise in the level of intracellular cyclic AMP with a selective increase in transepithelial Na^+ transport. However, such functional diversity among principal cells appears unlikely, because recordings of the basolateral membrane voltage from a single cell (Fig. 8) show that it responds in characteristic fashion to both Anoga-DH_{31} and Anoga-DH_{44}.

Secondly, a quantitative difference in the cyclic AMP signal obtained in response to Anoga-DH_{31} and Anoga-DH_{44} could determine the extent to which Na^+ transport is stimulated. In this context, it is worth noting that the CRF-related diuretic hormone of *D. melanogaster* (Drome-DH_{44}) stimulates cyclic AMP production by Malpighian tubule principal cells and also activates a cyclic AMP-specific phosphodiesterase (PDE), which will curtail the second messenger response (Cabrero et al., 2002). This was not investigated in the present study, which measured cyclic AMP production by Malpighian tubules after PDE activity was inhibited by IBMX. However, a quantitative difference in cyclic AMP signalling is unlikely to explain why only Anoga-DH_{31} has natriuretic activity, because Anoga-DH_{44} does not selectively stimulate Na^+ transport by Malpighian tubules in which PDE activity was inhibited by IBMX.

The third possibility to be examined was whether the activation of both paracellular and transcellular pathways by Anoga-DH_{44} (see above) militates against a natriuretic response. This also appears unlikely because Anoga-DH_{31} selectively stimulates transepithelial Na^+ transport by Malpighian tubules in which the paracellular pathway is first activated by Musdo-K. Similarly, differences in signalling exist for β_2 and β_1 adrenergic receptors in vertebrate heart tissue, which are believed to be due to differences in compartmentation of cyclic AMP in the tissue. Three principles of compartmentation of cyclic AMP signalling have been postulated: not all cyclic AMP gains access to all protein kinase A; not all protein kinase A has access to all possible substrates; and not all cyclic AMP has access to all cellular phosphodiesterases (Steinberg and Brunton, 2001). There is evidence that most β_2 adrenergic receptors are localized in caveolae, whereas most β_1 adrenergic receptors are not; this difference in localization in the plasma membrane may explain differences in the cyclic AMP response observed on stimulation of these receptors (Steinberg and Brunton, 2001). It is likely that the receptors for the CRF-like DH, *vs* the CT-like DH, are localized differently in the cell membrane, giving rise to differences in their signalling. It is also possible that these receptors couple to different G proteins; Culsa-DH leads to an apparent activation of both intracellular Ca^{2+} and cyclic

AMP, depending on the concentration of the ligand (Clark et al., 1998b). By contrast, our data point to Anoga-DH_{31} leading to only elevation of cyclic AMP.

In conclusion, the diuretic and natriuretic activities of Anoga-DH_{31}, a CT-like diuretic hormone common to *An. gambiae* and *Ae. aegypti*, are consistent with it being the MNP identified by Petzel et al. (1985). However, we have yet to show that Anoga-DH_{31} is released into the circulation when the insect takes a blood meal.

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