# The *Dh* gene of *Drosophila melanogaster* encodes a diuretic peptide that acts through cyclic AMP

Pablo Cabrero[1], Jonathan C. Radford[1], Kate E. Broderick[1], Laurence Costes[2], Jan A. Veenstra[2],
Eric P. Spana[3], Shireen A. Davies[1] and Julian A. T. Dow[1],*

[1]*IBLS Division of Molecular Genetics, University of Glasgow, Glasgow G11 6NU, UK*, [2]*Laboratoire de
Neuroendocrinologie des Insectes, Université Bordeaux I, Avenue des Facultés, 33405 Talence Cedex, France* and
[3]*Syngenta Biotechnology, Inc., Research Triangle Park, NC 27709, USA*

*Author for correspondence: j.a.t.dow@bio.gla.ac.uk

## Summary

*Dh*, the gene that encodes a CRF-like peptide in *Drosophila melanogaster*, is described. The product of this gene is a 44-amino-acid peptide (Drome-DH$_{44}$) with a sequence almost identical to the *Musca domestica* and *Stomoxys calcitrans* diuretic hormones. There are no other similar peptides encoded within the known *Drosophila* genomic sequence. Functional studies showed that the deduced peptide stimulated fluid production, and that this effect was mediated by cyclic AMP in principal cells only: there was no effect on the levels of either cyclic GMP or intracellular calcium. Stimulation also elevated levels of cyclic AMP (but not cyclic GMP) phosphodiesterase, a new mode of action for this class of hormone. The transcript was localised by *in situ* hybridisation, and the peptide by immunocytochemistry, to two groups of three neurones in the pars intercerebralis within the brain. These cells also express receptors for leucokinin, another major diuretic peptide, implying that the cells may be important in homeostatic regulation.

Key words: neuropeptide, Malpighian tubule, corticotropin-releasing factor (CRF), cyclic AMP, *Drosophila melanogaster*.

## Introduction

Several insect peptides have significant sequence similarity to those of vertebrates. One such is the corticotropin-releasing factor (CRF)-like insect diuretic peptide family, described in 15 species of insect to date (Audsley et al., 1995; Baldwin et al., 2001; Clark and Bradley, 1998; Clark et al., 1998a,b; Clottens et al., 1994; Coast and Kay, 1994; Furuya et al., 2000, 1998). In locust, as in all insects studied so far, CRF diuretic-related peptides stimulate fluid secretion *via* cyclic AMP (cAMP) (Coast et al., 1991). The ultimate target of cAMP signalling is thought to be the apical V-ATPase (Clark et al., 1998b).

We describe in this work the *Dh* gene that encodes Drome-DH$_{44}$, the first member of the CRF-like family to be found in *Drosophila melanogaster*. Functional analysis confirms that this peptide is active on the Malpighian (renal) tubule, and acts through cAMP. The peptide is expressed in six neuroendocrine cells in the pars intercerebralis. We also show for the first time that CRF directly activates its cognate phosphodiesterase, so limiting its own signal.

## Materials and methods

### Materials

Neuropeptide Drome-DH$_{44}$ was synthesised at the Emory University Microchemical Facility in Atlanta, GA, USA. Schneider's medium was obtained from Gibco Life Technologies. *Drosophila* saline (pH 6.7, in mmol l$^{-1}$, NaCl, 117.5; KCl, 20; CaCl$_2$, 2; MgCl$_2$, 8.5; NaHCO$_3$, 10.2; NaH$_2$PO$_4$, 4.3; Hepes, 15; Glucose, 20) was made using products from Sigma, Fisher Scientific and BDH Laboratory supplies. Zaprinast (cyclic GMP-dependant phosphodiesterase inhibitor) was obtained from Calbiochem. IBMX (non-specific phosphodiesterase inhibitor) was purchased from Sigma. Cyclic GMP (cGMP) and cAMP RIA kits (Amerlex-M) were obtained from Amersham Pharmacia plc. Coelenterazine was obtained from Molecular Probes and dissolved in ethanol before use. Primers designed for sequencing were purchased from MWG Biotech AG. All other chemical products were acquired from Sigma.

### Drosophila *methods*

*Drosophila melanogaster* Meig were maintained in a 12 h:12 h light:dark cycle on standard corn meal-yeast-agar medium at 25°C. Strain Oregon R (wild type), c42-aeq and c710-aeq fly lines were those described previously (O'Donnell et al., 1998; Rosay et al., 1997; Terhzaz et al., 1999). Briefly, c42-aeq and c710-aeq are lines that express an aequorin gene under the control of the yeast UAS$_G$ promoter and appropriate

P{Gal4} insertions, which drive expression in principal and stellate cells of the main segment, respectively (Sözen et al., 1997).

### Fluid secretion assays

Fluid secretion assays were performed as described previously (Dow et al., 1994). Malpighian tubules from adult flies were dissected under Schneider's medium and isolated into $10\,\mu l$ drops of a 1:1 mixture of Schneider's medium:*Drosophila* saline. All values are means ± S.E.M.

### Measurement of intracellular cyclic nucleotide concentrations

Intracellular cAMP and cGMP concentrations were measured by radioimmunoassay as described previously (Davies et al., 1995). Briefly, 20 tubules per sample were dissected under Schneider's medium and incubated for 10 min in the presence of $10^{-4}\,mol\,l^{-1}$ isobutyl methyl xanthine (IBMX) for cAMP measurements and $10^{-4}\,mol\,l^{-1}$ zaprinast for cGMP measurements, prior to stimulation with $10^{-7}\,mol\,l^{-1}$ Drome-DH$_{44}$ for a further 10 min. The reaction was interrupted with ice-cold ethanol and samples homogenised, dried and resuspended in $0.05\,mol\,l^{-1}$ sodium acetate buffer before being assayed. Samples were assayed for cyclic nucleotide content by competitive radioimmunoassay following the manufacturer's instructions (Amersham Pharmacia, plc).

### Measurement of intracellular calcium

The effect of Drome-DH$_{44}$ on intracellular $[Ca^{2+}]$ levels in Malpighian tubules was assessed using a P{Gal4}/UAS-Aequorin system as described previously (MacPherson et al., 2001; O'Donnell et al., 1998; Rosay et al., 1997; Terhzaz et al., 1999). Briefly, Malpighian tubules were dissected and incubated in Schneider's medium containing $2.5\,\mu mol\,l^{-1}$ coelenterazine for 3–4 h. Real-time luminescence was measured with a Berthold–Wallac luminometer, using 0.1 s sampling bins. The luminometer was equipped with dual injectors for addition of drugs. Analysis and back-integration of the results were performed with a Mac Perl program as described previously (Rosay et al., 1997).

### Phosphodiesterase assays

For each sample, 50 Oregon R tubules (20–30 μg of protein) were dissected into $50\,\mu l$ of PBS (pH 7.4) and homogenised. 2 ml of tritiated cAMP working stock ($0.185\,kBq\,ml^{-1}$ in $1\,mmol\,l^{-1}$ cAMP, $10\,mmol\,l^{-1}$ Tris, $5\,mmol\,l^{-1}$ MgCl$_2$, pH 7.4) were added to each sample, on ice. Blank samples were prepared using $50\,\mu l$ of PBS and $50\,\mu l$ of working stock; positive controls were made as for blanks except that PBS was replaced by pCDNA-bovPDE4 transformed cell lysates, to provide a positive control for phosphodiesterase-4 (PDE4) activity (Corbin et al., 2000). The reaction mixtures were incubated at 30°C for 10 min and then terminated by boiling for 2 min. Samples were chilled on ice and incubated for 10 min with $25\,\mu l$ of $1\,mg\,ml^{-1}$ *Ophiophagus hannah* 5'-nucleotidase (Sigma) to allow conversion of guanosine monophosphate to guanosine. $400\,\mu l$ of resuspended Dowex 1-Cl resin, (Sigma) (1:2 v/v, in water) was added to each sample and vortexed briefly three times at 5 min intervals. The tubes were centrifuged at $12\,000\,\boldsymbol{g}$ for 2 min and $150\,\mu l$ of the supernatant removed and added to 2 ml Optiflow scintillant. The samples were counted for 60 s and PDE activities calculated using a standard formula (Corbin et al., 2000). Final activity was expressed per mg protein, by dividing by the amount of protein in the sample assayed. Protein concentrations were assayed according to standard protocols (Lowry, 1951).

Assays for tubule cG-PDE activity were carried out as for cA-PDE assays, except that tritiated cGMP working stock was used ($0.185\,kBq\,ml^{-1}$ in $1\,mmol\,l^{-1}$ cGMP, $10\,mmol\,l^{-1}$ Tris, $5\,mmol\,l^{-1}$ MgCl$_2$, pH 7.4) and positive controls carried out using pCDNA-bovPDE5 transformed cell lysates (Corbin et al., 2000).

### Localisation of expression of the Dh gene
#### Antibodies

Approximately 2 mg of Drome-DH$_{44}$ was conjugated to 5 mg of thyroglobulin using difluorodinitrobenzene as the conjugating agent, as described elsewhere (Kean et al., 2002). A single New Zealand white female received a total of four injections at 5–6 week intervals. The first injection was performed with complete Freund's adjuvant and for the subsequent injections incomplete Freund's adjuvant was used. The rabbit was bled 10 days after each booster. Three antisera to other insect CRF-like diuretic hormones were used, the N- and C-terminal specific antisera to *Manduca sexta* diuretic hormone (Veenstra and Hagedorn, 1991) and a previously unpublished antiserum to *Culex salinarius* CRF-like diuretic hormone. The latter was produced by J.A.V. while at the University of Tucson using the same protocol as that described here.

#### Immunocytochemistry

The protocol used for peptide immunocytochemistry was the same as that described recently (Kean et al., 2002). The third and fourth bleedings of the rabbit used to raise antiserum to Drome-DH$_{44}$ gave very similar results and were used at 1:2000 dilution. The antisera to the other insect CRF-like diuretic hormones were used at a dilution of 1:500. For double-labelling experiments the anti-CRF-like antibodies were purified from the serum by octanoic acid precipitation, dialyzed, lyophilized and labelled with carboxytetramethylrhodamine, as described elsewhere (Veenstra et al., 1995). For double labelings the tissues were first incubated with the leucokinin receptor antibody (Radford et al., 2002) at a dilution of 1:1000, followed by a fluorescein-labeled Fab fragment of goat-anti-rabbit IgG (Jackson Immunologicals) to visualize the leucokinin receptor antibody, and then the rhodamine-labeled IgGs to CRF-like diuretic hormone, following a protocol described elsewhere (Veenstra et al., 1995).

#### In situ *hybridisation*

Adult and larval brains were dissected, fixed for 25 min in

5% paraformaldehyde in PBT (PBS with 0.1% Tween 20), washed five times in PBT and digested for 9 min in 1 ml PBT containing 4 µg ml$^{-1}$ proteinase K. The digestion was stopped by washing the tissue twice in 1 ml cold 0.2% glycine in PBT under agitation, and once in PBT. The tissue was then fixed again for 25 min in 5% paraformaldehyde in PBT, washed five times in PBT, once with a 1:1 mixture of PBT and hybridisation solution, and thrice in hybridisation solution. The hybridisation solution consisted of 50% formamide, 5× SSC, 100 µg ml$^{-1}$ heparin, 100 µg ml$^{-1}$ denatured salmon sperm DNA and 0.1% Tween 20 in diethyl pyrocarbonate-treated distilled water (Sambrook and Russell, 2001). After prehybridisation for 1 h at 55°C, digoxygenin-labelled RNA probes were added and allowed to hybridise overnight. Digoxygenin-labelled RNA probes were prepared using a commercial kit from Roche Molecular Biochemicals following the instructions of the manufacturer. The probe was reduced by alkaline treatment to a size of approximately 300 nucleotides. After washing the tissue five times for 15 min at 55°C in hybridisation solution, tissues were washed at room temperature, once in 1:1 mixture of hybridisation solution and PBT, and four times in PBT. An alkaline-phosphatase-labelled digoxygenin antibody was used to localise the probes.

### Inmunocytochemistry for cyclic AMP

Slides were treated with 100 µl of 0.1 mg ml$^{-1}$ poly-L-lysine solution for 30 min, washed with water and left to dry. Tubules were dissected in Schneider's *Drosophila* medium and stuck onto slides in 1× PBS solution. A solution with final concentration $10^{-7}$ mol l$^{-1}$ DromeDH$_{44}$ and $10^{-5}$ mol l$^{-1}$ IBMX (phosphodiesterase inhibitor) in 1× PBS was added for 7 min (the same solution without peptide was added to control samples). The tubules were fixed with 4% paraformaldehyde in 1× PBS for 30 min. They were washed 5 times in 1× PBS before permeabilization with 0.2% (v/v) Triton X-100 in 1× PBS for 30 min. Permeabilization solution was changed every 10 min, after which the tubules were blocked for 3 h in PAT [PBS containing 0.5% (w/v) Sigma cold fraction V bovine serum albumin and 0.2% (v/v) Triton X-100]. The tubules were then hybridised overnight in a humidity chamber with the primary antibody [rabbit anti-cyclic AMP polyclonal antiserum (US Biological C8450)], at 1:250 dilution in PAT. Tubules were then blocked with PAT containing 2% (v/v) normal rabbit serum for 2 h. Samples were incubated for 1 h with the secondary antibody (fluorescein-conjugated anti-rabbit) diluted 1:250 in PAT with 2% normal rabbit serum in a humidity chamber. The tubules were then washed 4 times over a period of 2 h in PAT and twice for 10 min in PBS before being mounted in VectaShield medium (Vector labs). Slides were examined under epifluorescence (Zeiss) and photographs were taken with an Axiocam HRc (Zeiss) using appropriate filters, and the same exposure time for all the samples, at 40×/0.75 magnification.

### Stastical analysis

Where appropriate, statistical significance of differences was assessed with Student's *t*-test (two-tailed) for unpaired samples, taking the critical level for $P=0.05$. Significant differences are marked graphically with an asterisk.

## Results

### Identification of the gene encoding Drome-DH$_{44}$

The *Stomoxys calcitrans* DH$_{45}$ CRF-like peptide was used as a 'bait' for a TBLASTN search that was run against the Berkeley *Drosophila* Genome database. The results were hits against three EST sequences: AT 14664 (GenBank accession no. BF499889), GH 27214 (GenBank accession no. AI514143) and HL08001 (GenBank accession no. AA699224), and genomic sequence at region 85D-E of chromosome 3R. This locus had been annotated automatically by the *Drosophila* genome project, and was subsequently identified *in silico* as containing a CRF peptide, and named *Dh* (Vanden Broeck, 2001). AT14664 and GH27214 (Research Genetics) were sequenced partially on the positive strand: GH27214 was found to be chimaeric, and so was rejected. The AT14664 clone was accordingly chosen for full sequencing due to its greater chance of carrying the complete cDNA of the gene. It was also chimaeric, but contained an apparently full-length cDNA for *Dh* with a poly(A) tail and a polyadenylation signal. This cDNA and deduced peptide is shown in Fig. 1A. This cDNA differs significantly from those annotated automatically by the genome project, or by previous workers. The automated Gadfly annotation misses the 5′ end of the gene and includes an intron between bases 1102–1103; and the recent automatic sequencing of the same cDNA by the genome project did not identify the chimaeric nature of the AT14664 clone. Accordingly, we believe that the cDNA sequence in Fig. 1 is authoritative. The cDNA has a poly(A) tail, with a polyadenylation signal centred 23 bases upstream of the first A. The prepropeptide contains only one region with similarity to any other genes, the area corresponding to the mature CRF-like peptide, which we named Drome-DH$_{44}$, nor are there any other obvious propeptide cleavage sites (Veenstra, 2000). Accordingly, we do not believe that other neuropeptides are encoded by this gene. The prepropeptide also has a signal peptide sequence, as identified with pSortII: this is required for peptides with a secretory fate. The sequence of the Drome-DH$_{44}$ peptide encoded by *Dh* is very similar to other known insect sequences, and is almost indistinguishable from other Dipteran sequences (Fig. 2). The Drome-DH$_{44}$ peptide sequence is flanked by dibasic convertase cleavage sites (Veenstra, 2000). The peptide is amidated, as are all the CRF-like peptides identified to date. Some studies suggest the importance of amidation for its function (Audsley et al., 1995).

The genomic context of *Dh* is shown in Fig. 1B. The gene is in a gene-dense area, and sits between *Scm*, an RNA polymerase II transcription factor, and CG9492, a dynein ATPase, within the genomic sequence. *Dh* is punctuated by four introns, one large enough to contain an entire gene (*frost*, encoding a cold-induced gene).

A

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            ><
      10        20        30        40        50        60        70        80        90       100
CTAATATCTACCAATTTATAGAACGCTCTGAATGATGAAAGCCACAGCGTGGTTTTGTCCGGTGTTATTAACTTTACTGTGTGCCACAAGGCTCGTCTGC
                  M   M   K   A   T   A   W   F   C   P   V   L   L   T   L   L   C   A   T   R   L   V   C>

     110       120       130       140       150       160       170       180       190       200
ACGGCCCAAAGAGGAGCAGTTGGAACAGGAGGAGCTGCAGGAGGATCGGGAGCGGCAGCGGGTGGAGCGGAGGTCGGCGGAAGCGGCCGCACAAACAGAT
    T   A   Q   R   G   A   V   G   T   G   G   A   A   G   G   S   G   A   A   A   G   G   A   E   V   G   G   S   G   R   T   N   R>
  ^

     210       220       230       240       250       260       270       280       290       300
ATCCTCTGGACTACCCGGATGGAACACGGAACTCACAGGATGACTTTCTGCTGGCCAAGAGGAACAAACGTCGCTGTCCATTGTCAATCCGCTGGATGT
    Y   P   L   D   Y   P   D   G   T   R   N   S   Q   D   D   F   L   L   A   K   R   N   K   P   S   L   S   I   V   N   P   L   D   V>
                                                                        ><

     310       320       330       340       350       360       370       380       390       400
GCTGCGCCAGCGGCTGCTCCTGGAAATCGCACGCAGGCAAATGAAGGAGAACTCCCGCCAGGTCGAACTGAATCGCGCCATCCTGAAGAACGTGGGCAAG
    L   R   Q   R   L   L   L   E   I   A   R   R   Q   M   K   E   N   S   R   Q   V   E   L   N   R   A   I   L   K   N   V   G   K>

     410       420       430       440       450       460       470       480       490       500
AGGGTCGTGCTGCGAGGAGGAGGAGGAGGCGGAGGATCGGGAGCAGGAGGATTGGCTCCCAAGGTGAGCAGGCGCTACCGCCAGCAGTGGCCAGTGGAGC
    R   V   V   L   R   G   G   G   G   G   G   G   S   G   A   G   G   L   A   P   K   V   S   R   R   Y   R   Q   Q   W   P   V   E>

     510       520       530       540       550       560       570       580       590       600
GGGAACTGGACGGGGAAAGGCAGAGGGAGAGGGAAAGGGAATGGGATGCAGTTCGTGAGGAGCAGCTGGACCGTCAACAGCTGCTGCCGTGGAAGCATTT
    R   E   L   E   R   E   R   Q   R   E   R   E   R   E   W   D   A   V   R   E   E   Q   L   D   R   Q   Q   L   L   P   W   K   H   F>

     610       620       630       640       650       660       670       680       690       700
CCCCAGCCAACTGTGGAGCTACGGATGGGCCCTGTCGCCGTACAAGGAGTCGTCACAGTTACAGTTTGCGGACTCCCAGCAATCAGCATCGACTGGACCA
    P   S   Q   L   W   S   Y   G   W   A   L   S   P   Y   K   E   S   S   Q   L   Q   F   A   D   S   Q   Q   S   A   S   T   G   P>

     710       720       730       740       750       760       770       780       790       800
CAATCCCAGGCACTCCCAAAGCAGTTGCAGTTGCTCAGCTATGCGAAGAAACCACTGGACGTGGCCGGAATGAGTCTGGCCAGGCATCGGGTCAGCGGAA
    Q   S   Q   A   L   P   K   Q   L   Q   L   L   S   Y   A   K   K   P   L   D   V   A   G   M   S   L   A   R   H   R   V   S   G>

     810       820       830       840       850       860       870       880       890       900
ATGAAGCCAACGAAACGAATCATGAGAACGACGATGGCAATGGGGCCAGTAAAAACCCGGCGAGATATGTGGATGACGGCGACAATGAGGGCGAGGATAG
    N   E   A   N   E   T   N   H   E   N   D   D   G   N   G   A   S   K   N   P   A   R   Y   V   D   D   G   D   N   E   G   E   D   S>

     910       920       930       940       950       960       970       980       990      1000
CTACAATGACGTGGGAACAGAAGGTGTGGGACTCGGCCTGGGAATGGGTGTGGGATTGGGTCTGGAACGGTTCGAGGTGCTGGAGGACAAGCCCAACTGG
    Y   N   D   V   G   T   E   G   V   G   L   G   L   G   M   G   V   G   L   G   L   E   R   F   E   V   L   E   D   K   P   N   W>

    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
GCCGATGAGGAACCAAACGAGTTGGTGGTGGTTAATGCAAATGACCGCGTGCCCTGGTCCTTTCCCTACCGCTTTCACAAAAGTCAGCATAACGTTAATT
    A   D   E   E   P   N   E   L   V   V   V   N   A   N   D   R   V   P   W   S   F   P   Y   R   F   H   K   S   Q   H   N   V   N>
><
    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
AATTACAGATATATGTAATACCGACACACATACCGATACCGATACCGAAACGGATACGATGCGATAGGGGCACCCAACGGCTTCTGAGTGCTGGAAGGTT

    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
ATTAATGCAAATCGACGGTTCCTAATGCGTGGCTACAAGGCAGGCATGTTGTGTGTGCACATACTCCCTTGATGACAAACTTACTCCCACGTTGCTAGAT

    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
GTACTCGCATTTGAACTCTATTTTATCGAAAATAACCAACGGTGTATGTAACGATTTATGCTACCTCTCTCACAGATACTGTTGAAAATAAAGTCCCCGA

    1410      1420      1430
TTAATGTGAAACAAAAAAAAAAAAAAAAAAAAAAAA
```

B



Fig. 1. The *Drosophila melanogaster* CRF-like gene. (A) cDNA and deduced peptide sequence of *Dh*. Putative dibasic convertase cleavage sites are underlined. The C-terminal amidation signal glycine residue is in italic. The positions of introns in the genomic sequence are marked with ><. The predicted peptide is indicated in blue. The encoded peptide has a signal peptide, with a predicted cleavage site between amino acid residues 24 and 25, marked with ^, as predicted by PSORTII Prediction program (http://psort.nibb.ac.jp/form2.html). The polyadenylation signal is indicated in yellow. Differences between the genomic sequence and the cDNA are marked in red. (B) Genomic context of *Dh*. View of region 85E of chromosome 3R. The neighbours of *Dh* are, on the negative strand, *Scm*, an RNA polymerase II transcription factor implicated in gene silencing, and CG9492, a dynein ATPase implicated in microtubule-based movement. Within an intron of *Dh*, on the opposite strand, is *frost* (*fst*), a gene implicated in response to cold. Dh (old) denotes the original, incomplete, Gadfly annotation of the Dh gene.



Fig. 2. Alignment of insect corticotropin-releasing factor-related peptides. The sequences producing significant alignments against Drome-DH$_{44}$ peptide after a protein Blast search are shown below in red. Mac Vector clustal analysis was performed to align the sequences. Residues highlighted in yellow have ≥50% identity. Swissprot accession numbers for each peptide are as follows: *Musca domestica* (house fly), DIUH_MUSDO; *Periplaneta americana* (American cockroach), DIUH_PERAM; *Zootermopsis nevadensis* (termite), DIUH_ZOONE; *Diploptera punctata* (Pacific beetle cockroach) class I, DIUH_DIPPU; *Locusta migratoria* (migratory locust), DIUH_LOCMI; *Tenebrio molitor* (yellow mealworm), DIU2_TENMO; *Acheta domesticus* (house cricket), DIUH_ACHDO; *Manduca sexta* (tobacco hornworm) precursor 1, DIU1_MANSE; *DromeDH, dh* gene product (present study); *Culex salinarus*, CCRF-DP.

### Effect of Drome-DH$_{44}$ on fluid secretion

Drome-DH$_{44}$ stimulates fluid secretion rates in *Drosophila melanogaster* Malpighian tubules at concentrations equal to or higher than $10^{-7}$ mol l$^{-1}$, typically by up to 1.5–2.5 nl min$^{-1}$ (Fig. 3A); this is comparable with stimulation rates observed for other native diuretic neuropeptides: DLK (Terhzaz et al., 1999), capa1, capa2 (Kean et al., 2002) in *Drosophila melanogaster*. The dose–response curve (Fig. 3B) shows that
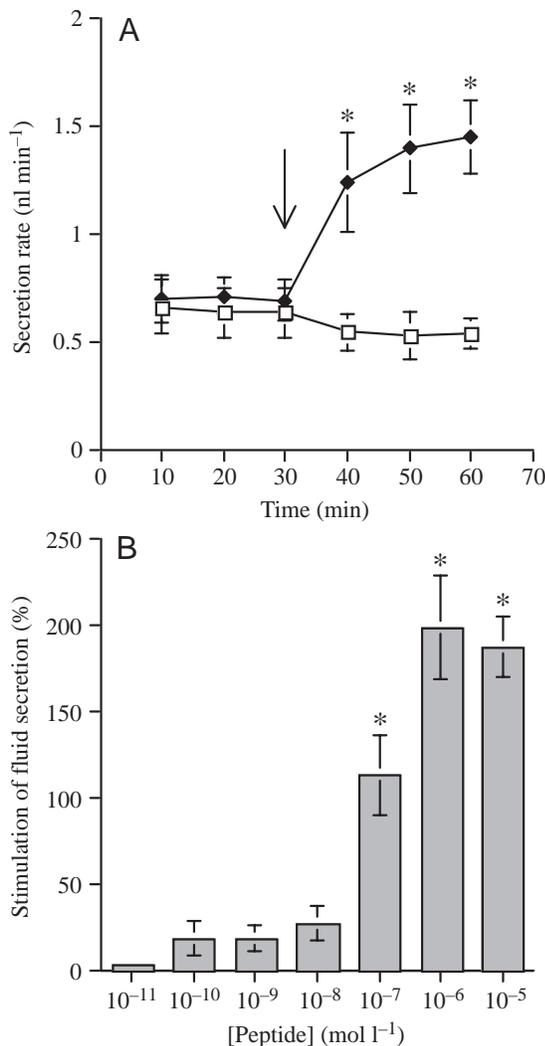


Fig. 3. Drome DH$_{44}$ increases fluid secretion rates. (A) Typical experiment, showing DromeDH$_{44}$-stimulated fluid secretion at $10^{-7}$ mol l$^{-1}$. The peptide was added at 30 min (arrow), and secretion rates measured as described in Materials and methods. Values are mean fluid secretion rates (nl min$^{-1}$) ± S.E.M. (*N*=6–9). Rates of DromeDH$_{44}$-stimulated secretion at 40–60 min differ significantly from control (asterisks; *P*<0.05). (B) Dose–response plot for Drome DH$_{44}$ stimulation of fluid secretion. Stimulated secretion rates were obtained from the typical assays shown in A. To normalise and compare data between different experiments, the results are expressed as the percentage increase of basal rate on peptide stimulation (maximal stimulated rate–mean basal rate)/basal rate×100) ± S.E.M. (*N*=7–9). Stimulation is significant (*P*<0.05) at concentrations above $10^{-8}$ mol l$^{-1}$.

the stimulation is significant when using concentrations greater than $10^{-8}$ mol l$^{-1}$.

### Cyclic nucleotide assays

In other species, CRF-like peptides have been shown to elevate intracellular cAMP levels. No cGMP increase was observed after stimulation with Drome-DH$_{44}$ (Fig. 4B). On the other hand, a clear response in cyclic AMP is shown after treatment, as expected for a CRF-like peptide (Fig. 4A). A significant increase, approximately 150% above the basal rate, is observed for concentrations over $10^{-8}$ mol l$^{-1}$, consistent with the effect on secretion (Fig. 3). Immunocytochemistry with an antibody against cAMP showed that the levels were selectively increased in principal cells only (Fig. 4C,D), consistent with models for *Drosophila* tubule function, in which electrogenic cation transport is performed by the principal cells, while stellate cells provide the main route for water and chloride fluxes (Dow and Davies, 1999, 2001; O'Donnell et al., 1996, 1998).

### Phosphodiesterase assays

For a second messenger to be plausibly implicated in a signalling pathway, there must be a mechanism for terminating the signal, as well as for generating it. In the case of cAMP, a phosphodiesterase is likely to be responsible. Tubules were stimulated with Drome-DH$_{44}$, and cAMP- and cGMP-directed phosphodiesterase activity separately measured.

Drome-DH$_{44}$ treatment doubles cAMP-PDE activity and halves cGMP-PDE activity, respectively, though the latter change is not significant (Fig. 5). Drome-DH$_{44}$ application thus directly stimulates the activity of the enzyme that breaks down its second messenger, and thus provides a feedback mechanism for limitation of the signal. This is the first time that this breakdown pathway has been shown to be modulated by an insect diuretic hormone.

### Intracellular calcium

It is conceivable that Drome-DH$_{44}$ could act indirectly to raise cAMP levels *via* intracellular calcium, or that it might act through calcium in other cell types. Accordingly, the transgenic aequorin system (Rosay et al., 1997) was used to seek a calcium correlate of hormone stimulation. The neuropeptides CAP$_{2b}$ (Davies et al., 1995) and DLK (Terhzaz et al., 1999) were used as positive controls to generate increases in calcium levels in principal and stellate cells respectively. As described previously, CAP$_{2b}$ and DLK produced elevated intracellular [Ca$^{2+}$] in principal and stellate cells, respectively, while Drome-DH$_{44}$ had no effect (Fig. 6).

### Localisation of expression of the CRF gene

Both immunocytochemistry (Fig. 7a,b) and *in situ* hybridisation (Fig. 7c) techniques mark a bilateral triplet of cells in the pars intercerebralis. The two antisera against the *Manduca sexta* peptide were not immunoreactive in *Drosophila,* while the two antibodies against the Dipteran hormones recognized the same cells, with the *Drosophila*
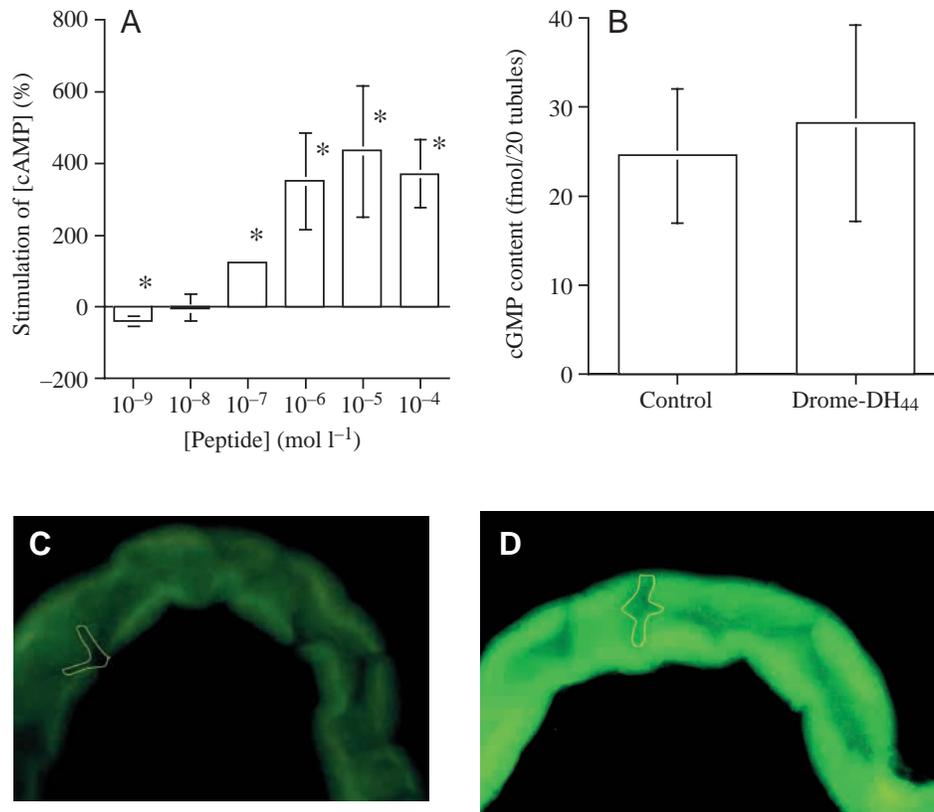
Fig. 4. Effect of Drome-DH$_{44}$ on cyclic nucleotide levels. (A) Drome-DH$_{44}$ elevates intracellular cAMP levels. Intracellular cAMP levels in wild-type (Oregon R) tubules were measured under control and Drome-DH$_{44}$ stimulated conditions by radioimmunoassay. The results are expressed as percentage increase of basal rate on peptide stimulation (stimulated rate–mean basal rate)×100/mean basal rate). Values are means ± S.E.M. ($N$=5–9;): stimulation of cAMP is significant ($P<0.05$) for concentrations above $10^{-8}$ mol l$^{-1}$. The mean basal cAMP level was 383±43 fmol/20 tubules. (B) cGMP levels are not affected by Drome-DH$_{44}$. Intracellular levels of cyclic GMP did not show a significant difference between control and Drome-DH$_{44}$ ($10^{-7}$ mol l$^{-1}$) stimulated conditions by RIA. The results are expressed as means ± S.E.M., $N$=4 independent samples, each pooled from 20 tubules. (C,D) Drome-DH$_{44}$-induced increase in cAMP is confined to principal cells. Immunocytochemistry of whole-mount tubules in control cells (C) and 10 min after application of $10^{-7}$ mol l$^{-1}$ Drome-DH$_{44}$ (D). Images were recorded under identical filter and exposure settings, using a matched-pair protocol. A typical stellate cell is outlined in each photograph.
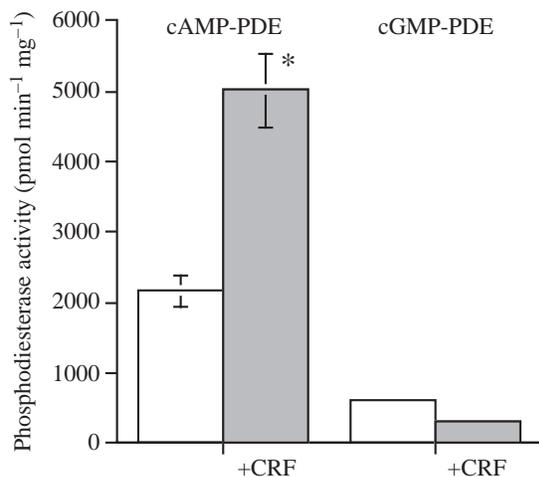


Fig. 5. Drome-DH$_{44}$ elevates cAMP-, but not cGMP-, phosphodiesterase. Values are means ± S.E.M., $N$=4 determinations. Where error bars are not shown, they are too small to be visible. *, $P<0.05$.

antibody being clearly more immunoreactive. In both adults and larvae, CRF-like diuretic hormone immunoreactivity was found exclusively in three bilateral pairs of cells in the pars intercerebralis, the axons which could be followed into the retrocerebral complex. These cells are very similar to the B-cells described from other flies (Panov, 1976). The morphology of these cells is thus typical of classical insect neuroendocrine cells. Using *in situ* hybridization the same number of cells was found in a closely similar location (Fig. 7c). No CRF-like immunoreactive cells were found in the abdominal ganglia, either by immunocytology or by *in situ* hybridisation. Interestingly, these cells also express Leucokinin receptor immunoreactivity, both in the cell bodies and the neurohaemal axons and axon terminals in the retrocerebral complex (Fig. 8).

## Discussion

The primary effect of insect diuretic peptides is considered to be stimulation of fluid secretion by Malpighian tubules.
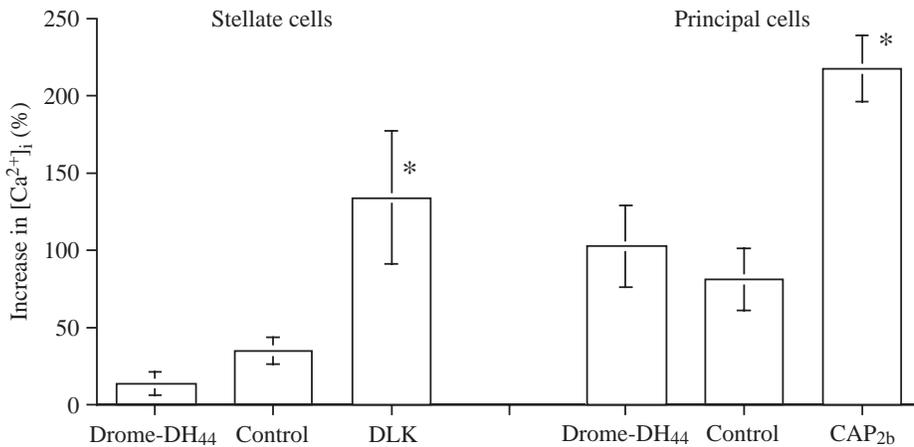
Fig. 6. Drome-DH$_{44}$ has no effect on intracellular calcium levels. $[Ca^{2+}]_i$ levels in real time were measured after stimulation with mock-injection (Control), CAP$_{2b}$, DLK and Drome-DH$_{44}$ at $10^{-7}$ mol l$^{-1}$. DLK and CAP$_{2b}$ are known to stimulate $[Ca^{2+}]_i$ levels in stellate and principal cells of the main segment, respectively, and were used as a positive control. To enable comparison between experiments, $[Ca^{2+}]_i$ values are expressed as mean percentage increases $\pm$ S.E.M. ($N$=4–8). Significant differences, relative to controls, are marked with an asterisk ($P<0.05$).
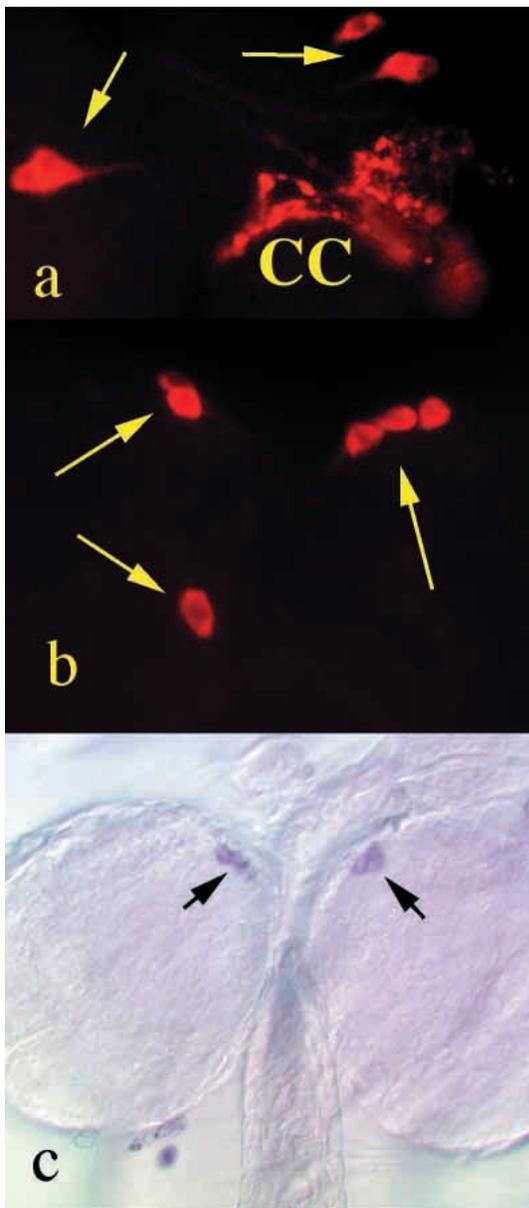
Three different families of insect neuropeptides have been shown to stimulate fluid secretion: CRF-like diuretic peptides, calcitonin-like peptides (Coast et al., 2001), the kinin family (Holman et al., 1984), and the CAP$_{2b}$ (Davies et al., 1995), capa-1 and capa-2 (Kean et al., 2002) family. Each class of peptides has a distinct mode of action: the capa-like peptides raise intracellular calcium levels and stimulate NOS to raise NO levels and thence cGMP levels; the kinins also act *via* intracellular calcium levels, to stimulate chloride shunt conductance (Hayes et al., 1989; O'Donnell et al., 1996, 1998; Pannabecker et al., 1993). Both calcitonin-like peptides and the CRF-like peptides are thought to act through cAMP. However, although CRF-like peptides have been reported previously to act through cAMP, this is the first demonstration that they have no effect on calcium. This may differ from the position in *Aedes aegypta* (another Dipteran), where CRF appears to have two effects: a very weak stimulation of secretion at low concentrations, which may be dependent on calcium, and a strong, cAMP-dependent stimulation of secretion at high concentrations, $>10^{-8}$ mol l$^{-1}$ (Clark et al., 1998a,b). In *Drosophila*, it appears that the response we describe here corresponds closely to the 'high-concentration' response described in *Aedes*. In addition, in *Drosophila*, Drome-DH$_{44}$ appears to raise cAMP levels only in the principal cells, implying a clear division of labour between hormones that act to stimulate electrogenic cation transport in principal cells (capa1, capa2, calcitonin-like peptide, CRF-like peptide), and hormones that act to raise chloride shunt conductance in stellate cells (leucokinins).

Expression of the CRF-like peptide appears to be rather



Fig. 7. CRF is expressed within a small set of neuroendocrine cells in the CNS. (a) Immunocytochemical localization of Drome-DH$_{44}$ in the larval nervous system. There are three cells (arrows) in each cerebral lobe with axons running to the corpus cardiacum (CC). (b) In the adult these six neuroendocrine cells are located in the pars intermedia. (c) *In situ* hybridization with antisense RNA identifies the same cells in the larval nervous system.
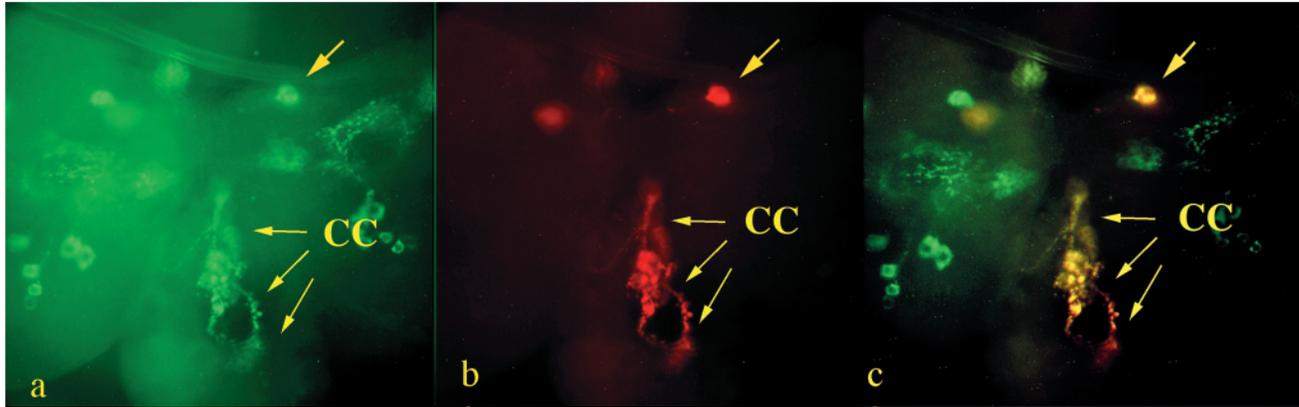
Fig. 8. Cells expressing *Dh* also express the leucokinin receptor. Larval brain, double stained for LKR, the *Drosophila* leucokinin receptor (Radford et al., 2002) and DromeDH$_{44}$. (a) Immunostaining for LKR (green), showing widespread expression; (b) immunostaining for DromeDH$_{44}$ (red), showing expression in cell triplets, as in Fig. 7 (not all visible in this plane of focus), and in their axons in the retrocerebral complex of the corpus cardiacum (CC); (c) merged image showing colocalisation.

limited, being confined to only a bilateral triplet of cells in the pars intercerebralis. The number of CRF-like peptide immunoreactive cells reported here is smaller than reported previously in the housefly *Musca domestica* (Iaboni et al., 1998) using an antiserum to locust CRF-like DH. Although we cannot exclude the possibility that the *Locusta* antiserum is more specific than the one we raised to the *Drosophila* hormone, we feel that this is unlikely, as both *in situ* hybridisation and immunocytochemistry techniques identify the same cells. Unlike in the moth *Manduca sexta* (Chen et al., 1994), the locust *Locusta migratoria* (Thompson et al., 1995) or the bug *Rhodnius prolixus* (Te Brugge et al., 2001), there are no abdominal neuroendocrine cells in *Drosophila* producing CRF-like diuretic hormones. This may be typical of Diptera, as in neither the mosquito *Aedes aegypti* (J.A.V., unpublished data) nor the housefly *Musca domestica* (Iaboni et al., 1998) have such abdominal neuroendocrine cells been found. In other species, some of these abdominal neuroendocrine cells express both leucokinins and CRF-like diuretic hormones, and it has been shown for the locust that these hormones have synergistic effects (Thompson et al., 1995). Although *Drosophila* has no neuroendocrine cells that produce both leucokinin and the CRF-like peptide, our observations suggesting expression of the leucokinin receptor in the CRF-like-peptide-producing neuroendocrine cells shows an alternative way of interaction between the two peptides; it is tempting to speculate that release of leucokinin into the haemolymph could induce and/or facilitate release of the CRF-like peptide.

In *Manduca sexta* the number of CRF-like-diuretic-hormone-producing neuroendocrine cells in the pars intercerebralis increases dramatically during metamorphosis. It had been suggested that this increase might be related to the very significant loss of water during metamorphosis when a watery caterpillar is transformed into a much lighter, flying moth (Veenstra and Hagedorn, 1991). The transformation of a maggot to a fly is similarly accompanied by a significant loss of water, but the number of the CRF-like-diuretic-hormone-producing neuroendocrine cells in *Drosophila* does not change during metamorphosis. It will be interesting to see whether this reflects a physiological functional difference of these hormones. Overall, it is not yet clear what role CRF-like peptides play in insect organismal physiology, although in *D. melanogaster* at least, a reverse genetic approach is conceivable. It is also interesting that the cDNA clone that we sequenced came from a testes library; although we have not addressed here the possibility of expression outside the CNS, it would be interesting to investigate whether there was any biological significance to this finding.

It is interesting to note that Drome-DH$_{44}$ does not act merely to raise cAMP levels, but also to stimulate cAMP-phosphodiesterase twofold. This change would have the effect of terminating the Drome-DH$_{44}$ signal even faster than would be expected if PDE levels remained constant, and would imply that Drome-DH$_{44}$ is primarily a short-term modulator of fluid secretion. Technically, we cannot separate the PDE responses of principal and stellate cells in these assays, although as the increase in cAMP concentration is confined to principal cells, it would be reasonable to suppose that the PDE response occurred at least in them. Although statistical significance was not achieved in these experiments (Fig. 5), there was an interesting downward trend in the cGMP-PDE assay, which would have the effect of potentiating stimulation by the capa peptides.

The similarity of action between Drome-DH$_{44}$ (this work) and calcitonin-like peptide (Coast et al., 2001) might at first seem puzzling. However, there is no guarantee that these peptides act exclusively on Malpighian tubules; it is quite possible that they have distinct actions elsewhere in the fly. The Malpighian tubule, as a prime site for homeostatic regulation, is charged with integrating inputs from a whole range of peptides, many of which may prove not to be primarily diuretic; and so it is not surprising to find multiple peptides with similar modes of action on this tissue (Dow and Davies, 2001; O'Donnell and Spring, 2000; Skaer et al., 2002).

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