Cockroach diuretic hormones: Characterization of a calcitonin-like peptide in insects

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Insect diuretic hormones are crucial for control of water balance. We isolated from the cockroach *Diploptera punctata* two diuretic hormones (DH), Dippu-DH_{31} and Dippu-DH_{46}, which increase cAMP production and fluid secretion in Malpighian tubules of several insect species. Dippu-DH_{31} and -DH_{46} contain 31 and 46 amino acids, respectively. Dippu-DH_{46} belongs to the corticotropin-releasing factor (CRF)-like insect DH family, whereas Dippu-DH_{31} has little sequence similarity to the CRF-like DH, but is similar to the calcitonin family. Dippu-DH_{46} and -DH_{31} have synergistic effects in *D. punctata* but have only additive effects in *Locusta migratoria*. Dippu-DH_{31} represents a distinct type of insect DH with actions that differ from those of previously identified insect peptides with diuretic activity.

Malpighian tubules | corticotropin-releasing factor | *Diploptera punctata* | *Locusta migratoria* | *Manduca sexta*

In insects, urine production by the Malpighian tubules (Mt) is driven by hormonally controlled active transport processes, rather than by ultrafiltration, as in vertebrates. There are several families of insect diuretic peptides, including myokinins, which increase urine production by elevating intracellular Ca^{2+} (1, 2), and the "corticotropin-releasing factor (CRF)-like" diuretic hormones (DH), which act via cAMP (3). CRF-like DH have been identified from eight species in five insect orders (4–14); they are similar to the sauvagine/CRF/urotensin I/urocortin family of vertebrate peptides.

For some years, only *Manduca sexta* was known to possess two CRF-like DH, Manse-DH (4) and Manse-DPII (9). Manse-DPII is shorter (30 residues) than other known DH and has lower sequence similarity with other DH than does Manse-DH. However, in 1998, we identified a second DH from *Tenebrio molitor*, termed Tenmo-DH_{47} (12) to distinguish this peptide from the known Tenmo-DH_{37} (11). More recently we identified two DH from *Hyles lineata* (a sphingid moth closely related to *M. sexta*), Hylli-DH_{30} and Hylli-DH_{41}, which each differ at only one residue from their *M. sexta* counterparts. Thus, in at least three insect species, two DH exist. The extent of sequence similarity between these "long" and "short" DH indicates that they belong to the same peptide family. However, they are most likely paralogous sequences (arising from a gene duplication event), as is the case for fish CRF and urotensin I. The lower potency of Tenmo-DH_{47} vs. Tenmo-DH_{37} (12) suggests for it a somewhat different role, just as urocortin, an orthologue of urotensin I (15), has effects that differ *in vivo* from those of CRF (16, 17).

We now report the identification of two DH from brain and corpora cardiaca (CC) of the Pacific beetle cockroach (*Diploptera punctata*), one of which (Dippu-DH_{31}) is a peptide with biological properties that differ from those of the CRF-like DH.

Materials and Methods

Insects. *D. punctata* were maintained as described previously (18). Brains and CC were dissected from adult males 2–10 days old. For bioassays, newly emerged adult males were isolated from the colony, and Mt were dissected (day 0). The *M. sexta* colony was reared essentially as described by Yamamoto (19). Newly emerged adult males (0–4 hr posteclosion) were chilled on ice for 15 min before dissection of Mt. *Schistocerca americana* were reared at 28°C on romaine lettuce with a 14-hr light/10-hr dark photoperiod. *Locusta migratoria*, taken from a colony at Birkbeck College, were reared under identical conditions but were fed fresh germinated wheat, with water provided ad libitum. The Mt were removed from decapitated 7- to 14-day-old adult female locusts.

Extraction of Brains and CC. Brain/CC complexes (1,040) were extracted as described earlier (11), but the solvent was methanol containing 9.9% CH_3CO_2H, 0.1% (vol/vol) 2-(methylthio)ethanol, 20 mM H_2SO_4, 0.1 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 0.01 mM pepstatin A, and 1.5 mg BSA. The mixture was additionally sonicated (5 min, 4°C). The supernatants from the extraction (11) were evaporated to half their volume and diluted with 200 ml of 0.1% trifluoroacetic acid (TFA).

Peptide Isolation. Extracts were purified by step elution with 0.1% TFA, 20% CH_3CN/0.1% TFA, 45% CH_3CN/0.1% TFA, and 60% CH_3CN/0.1% TFA through 5 g of Vydac (Hesperia, CA) C_4 reversed-phase packing material (11). To each fraction eluted, 5 mg of BSA was added. Bioassays with Mt of *M. sexta* and *S. americana* revealed that diuretic activity, as determined by secretion of cAMP, was confined to a fraction eluted with 45% (vol/vol) acetonitrile/0.1% TFA. This solution was diluted with water, loaded onto a Vydac C_{18} RP-HPLC column, and eluted as given in the legend to Fig. 1. Fractions A and B were separately diluted with water and injected onto a Polymer Laboratories (Amherst, MA) PLRP-S (5 μm, 100 Å, 2.1 mm × 150 mm) column eluted at 200 μl/min with a gradient of acetonitrile (4–61%) in 0.1% TFA. After locating active fractions A and B, these were separately purified on a Reliasil (Column Engineering, Ontario, CA) C_{18} column (5 μm, 300 Å, 1.0 mm × 150 mm) eluted at 50 μl/min with a gradient of acetonitrile (4–61%) in 0.1% TFA.

Peptide Microanalysis and Synthesis. Positive-ion electrospray ionization spectra of the samples were acquired as described (11)

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Abbreviations: CC, corpora cardiaca; CRF, corticotropin-releasing factor (corticoliberin); DH, diuretic hormone; DP, diuretic peptide; Mt, Malpighian tubule; TFA, trifluoroacetic acid.

Data deposition: The sequences reported in this paper have been deposited in the Swiss-Prot Database (accession nos. P82372 and P82373).

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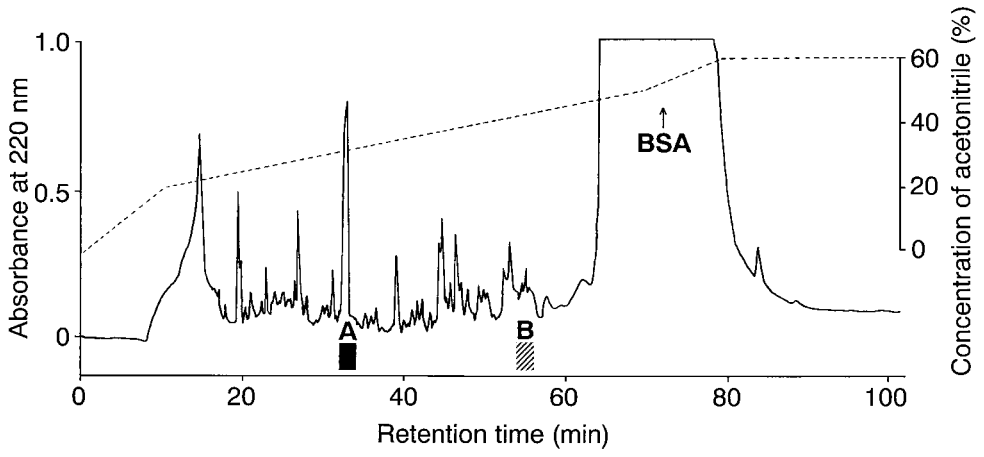

Fig. 1. Separation of *D. punctata* DH performed by RP-HPLC (5-μm Vydac 300-Å C_{18} column, 4.6 mm × 150 mm, eluted at 1.0 ml/min) with a gradient of aqueous acetonitrile (composition indicated by dashed line) with constant 0.1% (vol/vol) heptafluorobutyric acid. Two-milliliter fractions were collected; those at 32–34 min (A) and 54–56 min (B) had biological activity. The BSA added to the extraction solvent eluted as a broad peak (arrow).

with a Finnigan-MAT (San Jose, CA) SSQ-710 mass spectrometer with Analytica (Branford, CT) electrospray source. Automated Edman degradation was performed with a Porton Instruments (Tarzana, CA) PI 2090 sequencer, a PE Biosystems (Foster City, CA) 494HT sequencer, or a Hewlett–Packard G1005A sequencer. Purified Dippu-DH_{31} (≈150 pmol) and -DH_{46} (≈100 pmol) were digested with lysyl endopeptidase (Wako BioProducts, Richmond, VA) and endoproteinase Asp-N (Boehringer Mannheim), respectively, and fractionated by RP-HPLC, and fragments were analyzed by electrospray ionization-MS. The C-terminal amide functionality was determined from the M_r 721.3 ± 0.1 fragment (DFLESI-NH_2, from Dippu-DH_{46}) and the 1341.1 ± 0.1 fragment (HLMGLAAANYAGGP-NH_2, from Dippu-DH_{31}), which agree with the calculated M_r of the amidated forms (721.35 and 1340.65, respectively); the masses of the C-terminal free acid forms are 0.98 atomic mass unit higher. Both putative DH were synthesized by using a PE Biosystems model 431A synthesizer using fluorenylmethoxycarbonyl protocols as reported (12, 20).

Bioassay for Isolation. Samples containing 1–2 head equivalents were taken for *in vitro* (6) assays with Mt from *M. sexta* and *S. americana*. BSA (50 μg) was added to avoid loss of peptides. Aliquots were dried (vacuum centrifuge) and redissolved in 400 μl of saline (21) containing 0.5 mM 3-isobutyl-1-methylxanthine to inhibit phosphodiesterases. Batches of 10 Mt pieces (each ≈0.5 cm long) from *S. americana* or a single Mt fragment (≈1.0 cm) from *M. sexta* were added to 100-μl aliquots and incubated for 1 h. The cAMP released into 50 μl of medium was quantified as described (12).

Bioassay for Physiological Studies. Ramsay assays (22) were conducted with modifications. For *L. migratoria,* complete details have been described elsewhere (23). Diuretic activity was calculated as the difference between fluid secretion rates (Δ nl/min) measured before and after the addition of stimulants to the bathing fluid, with each tubule serving as its own control. To reduce between-assay variability when constructing dose-response curves, peptides were tested alongside control tubules stimulated maximally with 50 nM Locmi-DH, and results were expressed as a percentage of the maximal rate of secretion achieved by using Locmi-DH. All experiments were performed at 22°C ± 3°C. In contrast, for *D. punctata*, diuretic activity was calculated as the difference between fluid secretion rates (nl/min) measured before and after the addition of stimulants to the bathing fluid and is expressed as the percentage stimulation, with each tubule serving as its own control. All experiments were performed at room temperature.

Measurement of Urine K^+ and Na^+ Concentrations. Monovalent cation concentrations were measured by using a modified Corning flame photometer (model 410) as described (24). Urine K^+ concentrations were measured in samples of about 15 nl, whereas samples of about 100 nl were needed for Na^+ measurements. For unstimulated tubules, urine samples from 4–6 tubules were pooled for the measurement of Na^+ and K^+, but at high urine flow rates, both parameters could be measured on samples from 1 or 2 tubules.

Results

Isolation and Identification of the Peptides. We measured DH activity in extracts by following cAMP secreted by isolated Mt from adult *S. americana* and from adult male *M. sexta* (0 to 4 hr posteclosion). The secretion of cAMP by insect Mt is well known and permits rapid assay for biological activity.

Frozen brains and CC dissected from ≈1,000 of the *D. punctata* adult males were extracted and purified by solid phase extraction using C_4 silica packing, followed by RP-HPLC (11). Two fractions, A and B, (Fig. 1) stimulated cAMP production in Mt of *S. americana*. The level of cAMP released by Mt of *M. sexta* was elevated only by factor B. The Mt of *M. sexta* were more sensitive to factor B than those of *S. americana*; accordingly, we used *M. sexta* for bioassay of factor B in subsequent purifications. For factor A, Mt of *S. americana* were used. Active factors A and B were purified by using two more RP-HPLC separations; ≈0.5 nmol of each DH was obtained in pure form from 1,000 *D. punctata* brain and CC.

Electrospray ionization mass spectrometry showed DH from A and B to have M_r of 2,987.0 ± 0.2 and 5,322.0 ± 0.1, respectively. Edman microsequencing of intact peptides gave the complete sequence for factor B, but the C-terminal residue of factor A "washed off" in sequence analysis. We generated C-terminal fragments from both DH by digestion with specific proteases to generate smaller peptide fragments. These fragments were used to determine whether or not the C terminus was amidated and to identify the C-terminal amino acid of factor A. Mass spectral analysis of the two different C-terminal fragments yielded the following complete sequences: GLDLGLSRGF-SGSQAAKHLMGLAAANYAGGP-NH_2 and TGTGPSLS-IVNPLDVLRQRLLLEIARRRMRQTQNMIQANRDFLE-

A. CRF-like DH and some members of the vertebrate CRF superfamily

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h,r,d,eCRF -----SEEPPISLDL TFHLLREVLEMARAE QLAQQAH-----SNR KLMEII-NH2
catco-CRF2 -----SEEPPISLDL TFHLLREVLEMARAE QLVQQAH-----SNR KMMEIF-NH2
xenla-CRF  -----AEEPPISLDL TFHLLREVLEMARAE QIAQQAH-----SNR KLMDII-NH2
catco-UI   -----NDDPPISIDL TFHLLRNMIEMARNE NQREQAG-----LNR KYLDEV-NH2
urocortin  ------DDPPLSIDL TFHLLRTLLELARTQ SQRERAE-----QNR IIFDSV-NH2
sauvagine  ------QGPPISIDL SLELLRKMIEIEKQE KEKQQAA-----NNR LLLDTI-NH2

Manse-DH   ------RMPSLSIDL PMSVLRQKLSLEK-E RKVHALR---AAANR NFLNDI-NH2
Hylli-DH41 ------RMPSLSIDL PMSVLRQKLSLEK-E RKVQALR---AAANR NFLNDI-NH2
Musdo-DP   ------NKPSLSIVN PLDVLRQRLLLEIAR RQMKENT-RQVELNR AILKNV-NH2
Culsa-DP   --------PSLSIVN PLDVLRQRITLEMAR RQMRENT-RQVERNK AILREI-NH2
Peram-DP   ---TG-SGPSLSIVN PLDVLRQRLLLEIAR RRMRQSQ-DQIQANR EILQTI-NH2
Dippu-DH46 ---TG-TGPSLSIVN PLDVLRQRLLLEIAR RRMRQTQ-NMIQANR DFLESI-NH2
Locmi-DH   ---MG-MGPSLSIVN PMDVLRQRLLLEIAR RRLRDAE-EQIKANK DFLQQI-NH2
Achdo-DP   ----T-GAQSLSIVA PLDVLRQRLMNELNR RRMRELQGSRIQQNR QLLTSI-NH2
Tenmo-DH47 AGALGESGASLSIVN SLDVLRNRLLLEIAR KKAKEGA---NR-NR QILLSL-OH
Manse-DPII ---------SFSVNP AVDILQH----RYME K---VAQ-----NNR NFLNRV-NH2
Hylli-DH30 ---------SFSVNP AVEILQH----RYME K---VAQ-----NNR NFLNRV-NH2
Tenmo-DH37 -------SPTISITA PIDVLRKTWEQERAR KQMVK--------NR EFLNSLN-OH
Dippu-DH31 ------------GL DLGLSRGFSGSQAAK HLMGLAA-----ANY AGGP-NH2
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B. Calcitonins and *D. punctata* DH_{31}

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Chick      CASLSTCVLGKLSQE LHKLQTYPRTDVGAG TP-NH2
Eel        CSALSTCVLGKLSQE LHKLQTYPRTDVGAG TP-NH2
Salmon     CSNLSTCVLGKLSQE LHKLQTYPRTNTGSG TP-NH2
Human      CGNLSTCMLGTYTQD FNKFHTFPQTAIGVG AP-NH2
Rat        CGNLSTCMLGTYTQD LNKFHTFPQTSIGVG AP-NH2
Porcine    CSNLSTCVLSAYWRN LNNFHRFSGMGFGPE TP-NH2
Dippu-DH31 GLDLGLSRGFSGSQA AKHLMGLAAANY-AG GP-NH2
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Fig. 2. (*A*) Alignment of the CRF-related insect DH and some members of the vertebrate CRF superfamily, performed by using the program CLUSTAL W (41) on all known members of both families (Swiss-Prot Database). The default gap opening penalty (10) was used. The order of sequences returned by the program represents the program's estimation of phylogeny. Those members of the CRF superfamily shown are human, rat, dog, and horse CRF (h, r, d, eCRF), frog (*Xenopus laevis*) CRF (xenla-CRF), one sucker (*Catostomus commersoni*) CRF (catco-CRF2), sauvagine, sucker urotensin I (catco-U-I), and rat urocortin. Residues underlined are identical in four or more of the DH, ignoring the higher sequence identity within the CRF superfamily. Species abbreviations are as per NCBI/Swiss-Prot: *Periplaneta americana*, Peram; *Culex salinarius,* Culsa; *Acheta domesticus*, Achdo; *Musca domestica*, Musdo; *T. molitor*, Tenmo; and *H. lineata*, Hylli. See text for others. (*B*) Sequence alignment (CLUSTAL W) of calcitonin from chicken, Japanese eel, human, pig, rat, and salmon with Dippu-DH_{31}.

SI-NH_2. We named these peptides *D. punctata* DH 31 (Dippu-DH_{31}) and -46 (Dippu-DH_{46}). Both peptides were synthesized for structural confirmation and bioassay. Patel *et al*. (25) have presented "unequivocal evidence of a hormonal function" for the CRF-like DH from *L. migratoria* (Locmi-DH, Fig. 2, previously called *Locusta* DP). Although such evidence has not been explicitly provided for other members of the CRF-like DH, we feel justified in abbreviating these peptides as DH rather than DF (diuretic factor) or DP (diuretic peptide).

Dippu-DH_{46} is readily recognized as a member of the CRF-like DH family, with 83% identity to Peram-DH and 72% identity to Locmi-DH. However, Dippu-DH_{31} does not align with the CRF-like DH when the program CLUSTAL W is used. A BLAST search for sequence similarity revealed over 50 diverse proteins from a number of species with sequence identities from 15 to 8 of the 31 residues of Dippu-DH_{31}, but found no peptide hormones. A manual search of the Peninsula Laboratories catalog of bioactive peptides revealed similarity to the sequence of calcitonin, in particular to the unusual Pro-amide C terminus. Therefore, we searched Swiss-Prot for calcitonin from a number of species and aligned the sequences with Dippu-DH_{31} (six identities, or 19%) by using CLUSTAL W; the results are shown in Fig. 2*B*.

Biological Evaluation of the Synthetic DH: Second-Messenger Assays. Synthetic Dippu-DH_{31} stimulates cAMP production by Mt of *S. americana* in a dose-dependent manner (EC_{50} = 16 nM; maximal stimulation = 20 pmol of cAMP per tubule segment). Locmi-DH has a maximal stimulation of ≈100 pmol of cAMP on *S. americana* tubule segments, but Manse-DH has no detectable activity on these tubules despite its higher similarity to Locmi-DH (≈49% sequence identity) than to Dippu-DH_{31}. The latter has only four residues in common with Locmi-DH in our alignment (Fig. 2*A*). Dippu-DH_{31} has no effect on production of cAMP by Mt of *M. sexta*, although they are stimulated by all known insect CRF-related DH (3) except Tenmo-DH_{37} and -DH_{47} (11), neither of which is amidated.

Biological Evaluation of the Synthetic DH: Fluid Secretion Assays. *Response of* D. punctata *Mt to Dippu-DH_{31} and -DH_{46}.* Synthetic Dippu-DH_{31} and -DH_{46} were tested for diuretic activity by using Mt from *D. punctata*. Both peptides stimulate fluid secretion in this species in a dose-dependent manner, with Dippu-DH_{31} and -DH_{46} having EC_{50} values of 9.8 nM and 13 nM, respectively. However, the maximal response to Dippu-DH_{31} is only 41% of that for Dippu-DH_{46} (Fig. 3*A*). Chicken calcitonin also stimulates secretion by *D. punctata* Mt in a dose-dependent manner (Fig. 3*A*), with an apparent EC_{50} of ≈380 nM, and a maximal stimulation perhaps higher than that of Dippu-DH_{46}. However,

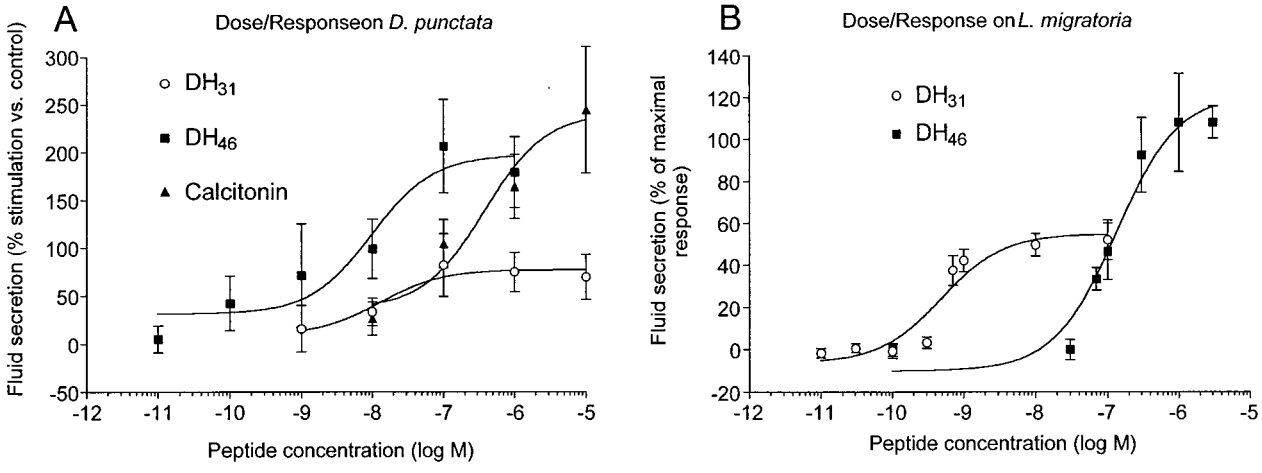

Fig. 3. (*A*) Dose–response curves for Dippu-DH_{31}, Dippu-DH_{46}, and chicken calcitonin with Mt of *D. punctata*. Fluid secretion was measured without and with hormone, and the percentage stimulation was determined. (*B*) Dose–response curve for diuretic activities of Dippu-DH_{31} and -DH_{46} with Mt of *L. migratoria*. Data points are the means and vertical lines the SEM of five to seven determinations. The data are expressed as percentages of the response to 50 nM Locmi-DH. All were fitted by nonlinear regression analysis by using PRISM 2.0b.

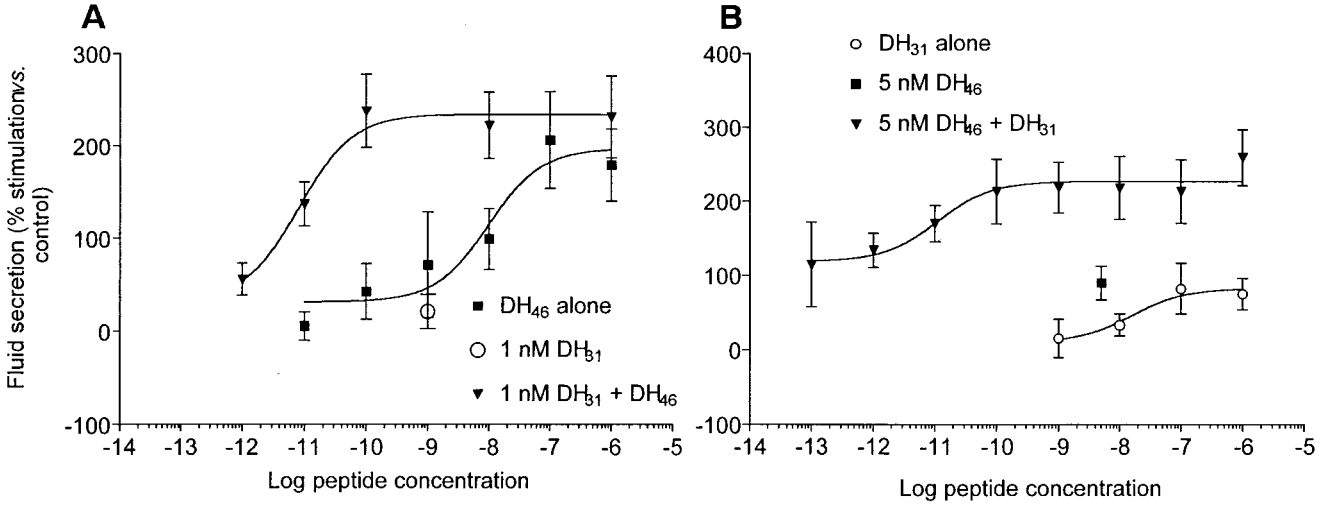

Fig. 4. Dose–response curve for fluid secretion by Mt from day 0 male *D. punctata* exposed to Dippu-DH_{46} and Dippu-DH_{31}, both separately and admixed. (*A*) Effect of Dippu-DH_{46} on cockroach Mt when tested alone (filled squares) or together with Dippu-DH_{31} (filled triangles); the effect of 1 nM Dippu-DH_{31} is shown with an open circle. (*B*) Response to Dippu-DH_{31} when assayed in the absence (open circles) or presence (filled triangles) of 5 nM Dippu-DH_{46}. A filled square shows the effect of 5 nM Dippu-DH_{46} by itself. Data points are the means and vertical lines the SEM of seven to nine determinations.

these data had much more scatter than data for the other peptides, and a more limited range of concentrations was tested.

A dose–response curve was determined for Dippu-DH_{46} (Fig. 4*A*) with Mt exposed also to 1 nM Dippu-DH_{31} (only ≈1/10 of the EC_{50} value); the EC_{50} for Dippu-DH_{46} when assayed together with Dippu-DH_{31} decreased to 8.3 pM, ≈1000-fold lower than its EC_{50} in Fig. 3*A*. Similarly, the dose–response curve for Dippu-DH_{31} determined in the presence of 5 nM Dippu-DH_{46} (Fig. 4*B*; a concentration roughly 1/3 of its EC_{50} value) gave a combined EC_{50} value of 11 pM, again ≈1000-fold lower than its EC_{50} in Fig. 3*A*. The total fluid secretion was in this case obviously higher than the summed secretion of the two peptides.

Response of L. migratoria *Mt to Dippu-DH_{31} and -DH_{46}.* Although Mt of the orthopteran *S. americana* were used to monitor purification of Dippu-DH_{31}, both this peptide and Dippu-DH_{46} were tested for activity on Mt of the related orthopteran *L. migratoria*, a far more characterized bioassay (3). Dippu-DH_{46} stimulated secretion maximally when compared with control tubules stimulated with Locmi-DH, and had an EC_{50} of 110 nM (95% confidence limits: 82–148 nM; Fig. 3*B*), a high concentration considering its 72% identity with Locmi-DH. On the other hand, Dippu-DH_{31} gave only 50% maximal stimulation, but had an EC_{50} of 0.56 nM (95% confidence limits: 0.51–0.64 nM; Fig. 3*B*).

When tested together at a variety of concentrations from threshold to EC_{50}, the activities of these two peptides were additive rather than synergistic (data not shown), a markedly different result from that obtained with *D. punctata* Mt. In certain of these experiments, urine samples were taken for analysis of monovalent cation concentrations. Consistent with stimulation of urine production, DH increase the rate of transport of K^+ and Na^+ into the tubule lumen, the driving force for fluid secretion. Dippu-DH_{46} has a greater effect on the transport of Na^+ than on K^+, and the urine $[K^+]$:$[Na^+]$ falls significantly ($P < 0.0001$) from 6.1 ± 0.3 ($n = 6$) in unstimulated tubules to 2.3 ± 0.3 ($n = 6$). In marked contrast, Dippu-DH_{31} has a nonselective effect on monovalent cation transport leaving the $[K^+]$:$[Na^+]$ ratio virtually unchanged (5.3 ± 0.3; $n = 6$). The decrease in the urine $[K^+]$:$[Na^+]$ ratio upon addition of Dippu-DH_{46} is consistent with the known action of Manse-DH on Mt of *M. sexta*. In this species, a Na^+-K^+-$2Cl^-$ cotransporter is stimulated by the action of this peptide at 5 nM concentration (21); a net influx of NaCl and KCl together would be expected to lower the $[K^+]$:$[Na^+]$ ratio if the apical cation transporters have a preference for Na^+ over K^+, as shown in *Rhodnius prolixus* Mt (26).

Assay of D. punctata *DH with pharmacological stimulants in Mt of* L. migratoria. To investigate the second-messenger systems involved in the response to the *D. punctata* DH, they were tested separately in combination with thapsigargin, an inhibitor of the endoplasmic reticulum Ca^{2+}-ATPase used to raise intracellular levels of calcium, and with 8-bromo-cAMP, a membrane-permeant cAMP analogue. The effects of thapsigargin (1 μM) and Dippu-DH_{31} (0.5 nM) were additive (Fig. 5*A*), whereas the Ca^{2+}-ATPase inhibitor acted cooperatively with Dippu-DH_{46} (200 nM), the combined response being significantly greater than the sum of their separate activities (Fig. 5*B*). In marked contrast, 8-bromo-cAMP (10 μM) produced an additive effect when tested with Dippu-DH_{46} (100 nM; Fig. 6*B*), but acted synergistically with 0.5 nM Dippu-DH_{31} (0.5 nM, Fig. 6*A*).

Interactions between diuretic peptides from L. migratoria *and* D. punctata *in Mt of* L. migratoria. Two diuretic peptides from *L. migratoria* are known: Locmi-DH (CRF-related) acts via cAMP

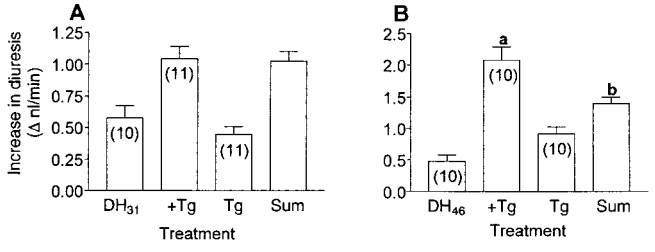

Fig. 5. In Figs. 5–8, bars represent the means and vertical lines the SEM for the number of determinations shown in parentheses. Fluid secretion is given as the change in fluid flow, with each tubule serving as its own control. Different letters indicate values that differ significantly. The effect of thapsigargin (*A*, 0.5 μM; *B*, 1 μM; shown as Tg on abscissa; "+Tg" means DH + Tg) on the rate of fluid secretion by *L. migratoria* tubules exposed to 0.5 nM Dippu-DH_{31} (*A*) and 200 nM Dippu-DH_{46} (*B*). Thapsigargin synergizes the effects of Dippu-DH_{46}, but not of Dippu-DH_{31}.

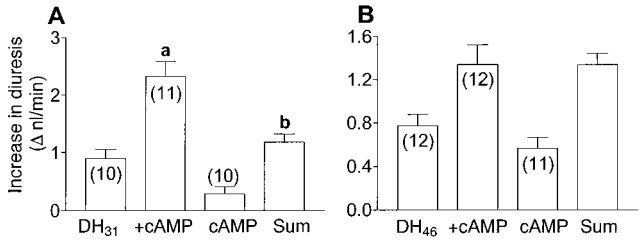

Fig. 6. The effect of 10 μM 8-bromo-cAMP (shown as cAMP on abscissa; ''+cAMP'' means DH + 8-bromo-cAMP) on the rate of fluid secretion by *L. migratoria* Mt exposed to 0.5 nM Dippu-DH_{31} (*A*) and 100 nM Dippu-DH_{46} (*B*). The cAMP analogue acts synergistically with Dippu-DH_{31}, but not with Dippu-DH_{46}.

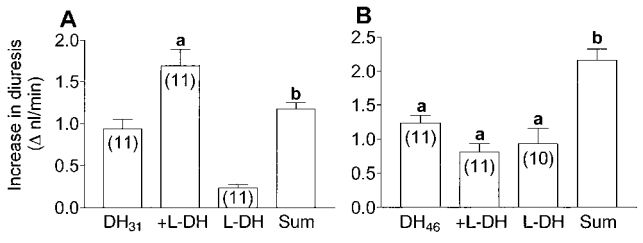

Fig. 8. The influence of Locmi-DH (*A*, 0.2 nM; *B*, 0.5 nM) on the rate of fluid secretion by *L. migratoria* tubules exposed to 0.5 nM Dippu-DH_{31} (*A*) and 300 nM Dippu-DH_{46} (*B*). Locmi-DH (shown as L-DH on abscissa; ''+L-DH'' means DH + Locmi-K) synergizes the effects of Dippu-DH_{31}, but not of Dippu-DH_{46}.

as second messenger, and locustakinin (Locmi-K), a myokinin (1), utilizes Ca^{2+} as second messenger. They act synergistically in stimulating urine production (24). Dippu-DH_{31} and -DH_{46} were tested in combination with one or the other of the *L. migratoria* peptides. The results are presented in Figs. 7 and 8. Dippu-DH_{31} acts synergistically with both Locmi-K (Fig. 7*A*) and Locmi-DH (Fig. 8*A*). On the other hand, Dippu-DH_{46} synergizes the effect of Locmi-K (Fig. 7*B*), but has no effect on the response to Locmi-DH (Fig. 8*B*).

Discussion

The sequences of Dippu-DH_{31} and -DH_{46} are remarkably different. Amino acid sequences of known insect CRF-like DH, plus sauvagine, urotensin I, urocortin, and CRF are shown in Fig. 2*A*. In this alignment, Dippu-DH_{46} is clearly a member of the CRF-related DH with high (83%) sequence identity to another cockroach DH, Peram-DP. However, Dippu-DH_{31} has very low similarity to other DH: only five residues are identical to those in Dippu-DH_{46} (Fig. 2*A*), and the C termini do not align. In the alignment shown in Fig. 2*B*, Dippu-DH_{31} has higher sequence identity to calcitonin (six residues identical with chicken and eel calcitonin, 19% sequence identity) than to any member of the CRF-like DH family of peptides. Dippu-DH_{31} lacks the disulfide bond between C-1 and C-7 of calcitonin; however, this disulfide ring is not important for biological activity of salmon calcitonin (27, 28). Moreover, we found chicken calcitonin to stimulate fluid flow in *D. punctata* Mt with EC_{50} of 380 nM (Fig. 3*A*), $\approx$40 times less potent than Dippu-DH_{31}, further strengthening the identity of Dippu-DH_{31} as a calcitonin-like peptide.

Dippu-DH_{31} and -DH_{46} stimulate cAMP production in Mt, an action characteristic of both ''long'' and ''short'' CRF-like DH. However, in the fluid secretion assays, there was a clear distinction between the actions of the two DH, lending support to the contention that Dippu-DH_{31} is not a CRF-like DH family member.

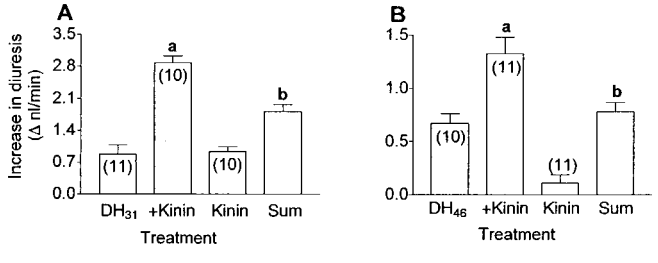

Fig. 7. The influence of Locmi-K (*A*, 0.2 nM; *B*, 0.1 nM; shown as kinin on abscissa; ''+kinin'' means DH + Locmi-K) on the rate of fluid secretion by *L. migratoria* Mt exposed to 0.5 nM Dippu-DH_{31} (*A*) and 200 nM Dippu-DH_{46} (*B*). Locmi-K synergizes the effects of both *D. punctata* DH.

Dippu-DH_{46} and Locmi-DH share 72% sequence identity and are identical in the region encompassing residues 7–11, previously implicated in signal transduction (29). Dippu-DH_{46} stimulates fluid secretion to the same extent as Locmi-DH in the locust diuretic assay, but the observed difference in EC_{50} value is high: this difference is consistent with the previously documented specificity of the *L. migratoria* receptor for binding (3). Dippu-DH_{46} and Locmi-DH differ most profoundly in a short region encompassing residues 29–35 (5 of the 6 residues differ). This region is a hypervariable region in the sequences of insect CRF-like DH and is believed to represent a loop region (30). The hypervariable region has little effect on the crossreactivity of the *M. sexta* DH receptor (3, 31), but may be important for receptor binding in *L. migratoria* and *Acheta domesticus* (3). The actions of Dippu-DH_{46} appear identical to those of Locmi-DH. Each acts additively with cAMP and synergistically with thapsigargin. Moreover, Dippu-DH_{46} promotes Na^+ transport, resulting in the urine $[K^+]$:$[Na^+]$ ratio being halved, and demonstrates synergism with Locmi-K, duplicating results obtained with Locmi-DH (24). The action of Dippu-DH_{46} may parallel that of Manse-DH in stimulating a Na^+-K^+-$2Cl^-$ cotransporter (21). The action of *Culex salinarius*-DP, or CCRF-DP, on Mt of a different mosquito species, *Aedes aegypti*, seems to constitute a different paradigm; at 1 nM concentration, it stimulates a paracellular Cl^- conductance, whereas at 100 nM it stimulates transcellular active Na^+ transport, together with passive Cl^- conductance (13, 32). This paradigm may reflect a mechanistic difference between the CRF-like DH in phytophagous vs. hematophagous insects. Interestingly, the effect of CCRF-DP at nanomolar levels is characteristic of the actions of the kinins, which elevate intracellular Ca^{2+}, whereas its actions at 100 nM are mimicked by dibutyryl-cAMP, typical of the other CRF-like DH.

Both Dippu-DH_{31} and -DH_{46} were isolated by monitoring elevation of secreted cAMP, but with synthetic replicates, both were characterized by using Ramsay assays, which test for actual diuretic activity. Assays were performed using Mt from both *D. punctata* and *L. migratoria*. Assays with cockroach tubules are extremely difficult, which is reflected in the higher SEM values seen in Fig. 3*A* (*D. punctata*) than in Fig. 3*B* (*L. migratoria*). We performed most studies on the actions of the two factors with *L. migratoria* Mt, because of the higher reproducibility of this assay and because Mt of *S. americana* were used for isolating Dippu-DH_{31}. Dippu-DH_{31} increases fluid secretion by $<$50% of the response obtained with Dippu-DH_{46} in Ramsay assays with both *L. migratoria* and *D. punctata* Mt. The two peptides are about equipotent (EC_{50}) on cockroach Mt, but, on locust Mt, Dippu-DH_{31} is $>$200 times more potent than Dippu-DH_{46}. The potency of Dippu-DH_{31} on locust Mt is consistent with the use of grasshopper (*S. americana*) Mt for the isolation of Dippu-DH_{31}, and suggests that orthopterans may use a similar DH as a circulating neurohormone. In marked contrast to the actions of Dippu-DH_{46}, Dippu-DH_{31} acts additively with thapsigargin and

synergistically with cAMP, results identical to those obtained previously with Locmi-K and other myokinins, although it lacks the characteristic C-terminal motif (FXXWG-NH_2) of that peptide family. Moreover, in common with Locmi-K, Dippu-DH_{31} synergizes the effects of CRF-like DH, notably Dippu-DH_{46}, in the cockroach assay, and synergizes the effects of Locmi-DH (but not of Dippu-DH_{46}) in the locust assay. This paradoxical ability of Dippu-DH_{31} to synergize the effects of Locmi-DH, but not Dippu-DH_{46}, in the locust probably reflects the high potency of Dippu-DH_{31} in this species (EC_{50} = 0.56 nM), whereas the potency of Dippu-DH_{46} is low (EC_{50} = 110 nM). The synergistic effect may be manifest only with a potent CRF-like DH; Locmi-DH has $EC_{50} \approx 1.7$ nM (25) in this species.

We hypothesize that Dippu-DH_{31} acts in a manner similar to the diuretic kinins, namely by using an increase in intracellular calcium to open a Cl^- conductance pathway. In support of this hypothesis, Dippu-DH_{31} has a nonselective effect on cation transport, leaving the urine $[K^+]$:$[Na^+]$ ratio unchanged, as previously reported for Locmi-K and very different from the effect of CRF-like DH (see above). The differing molecular action would account for differences in the effects of Dippu-DH_{31} and -DH_{46} in the diuretic assays, and for their synergistic actions on *D. punctata* Mt. The synergistic effects of Dippu-DH_{31} and Locmi-K in the *L. migratoria* bioassay are harder to rationalize. In this context, it is noteworthy that Dippu-DH_{31} stimulates cAMP production in grasshopper Mt, the bioassay used for its isolation, whereas Locmi-K has no effect on this second messenger pathway.

To account for the apparent paradox between the elevation by Dippu-DH_{31} of cAMP in second messenger assays vs. its apparent action via Ca^{2+} in the fluid secretion assays, it is significant that several peptide hormones, including pituitary adenylyl cyclase activating peptide (PACAP) (33–36), calcitonin (37–39), and adipokinetic hormone (40), can elevate *both* the adenylyl cyclase and phosphoinositide pathways in their target tissues. Cell lines expressing a single, defined, recombinant calcitonin receptor show elevation of both cytosolic cAMP and Ca^{2+} upon ligand binding (38, 39). Furthermore, calcitonin has been shown to have cell cycle-specific effects in porcine kidney cells (LLC-PK1), elevating intracellular cAMP during the G_1 phase, but activating the protein kinase C pathway in the S phase (37).

In conclusion, we have identified two DH from *D. punctata* that differ both in their sequences and in their mode of action. Dippu-DH_{46} is clearly a CRF-like DH and, like other peptides of this family, acts via a cAMP-dependent mechanism to stimulate cation (mainly Na^+) transport. On the other hand, Dippu-DH_{31} appears to be a different type of peptide, more similar to vertebrate calcitonin than to CRF-like DH. Dippu-DH_{31} no doubt acts at receptors separate from those responding to Dippu-DH_{46} and appears to stimulate fluid secretion by a Ca^{2+}-dependent mechanism.

Note. The *Drosophila melanogaster* genome (42) encodes a peptide (TVDFGLARGYSGTQEAKHRMGLAAANFAGGP-NH_2) that is 71% identical with Dippu-DH_{31}, 87% if allowing for conservative substitutions. The sequence is preceded and followed by proper processing sites. Laenen (43) partially sequenced a putative diuretic peptide from the ant *Formica polyctena*. This peptide is identical with Dippu-DH_{31} in its 29 N-terminal residues, but the molecular mass is lower by 28 Da, showing it has a different C terminus. It would appear, therefore, that calcitonin-like DH are widespread in the Insecta.

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1. Coast, G. M. (1996) *Peptides* **17,** 327–336.
2. O'Donnell, M. J., Dow, J. A. T., Huesmann, G. R., Tublitz, N. J. & Maddrell, S. H. P. (1996) *J. Exp. Biol.* **199,** 1163–1175.
3. Audsley, N., Kay, I., Hayes, T. K. & Coast, G. M. (1995) *Comp. Biochem. Physiol. A Physiol.* **110A,** 87–93.
4. Kataoka, H., Troetschler, R. G., Li, J. P., Kramer, S. J., Carney, R. L. & Schooley, D. A. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 2976–2980.
5. Lehmberg, E., Ota, R. B., Furuya, K., King, D. S., Applebaum, S. W., Ferenz, H.-J. & Schooley, D. A. (1991) *Biochem. Biophys. Res. Commun.* **179,** 1036–1041.
6. Kay, I., Coast, G. M., Cusinato, O., Wheeler, C. H., Totty, N. F. & Goldsworthy, G. J. (1991) *Biol. Chem. Hoppe-Seyler* **372,** 505–512.
7. Kay, I., Wheeler, C. H., Coast, G. M., Totty, N. F., Cusinato, O., Patel, M. & Goldsworthy, G. J. (1991) *Biol. Chem. Hoppe-Seyler* **372,** 929–934.
8. Kay, I., Patel, M., Coast, G. M., Totty, N. F., Mallet, A. I. & Goldsworthy, G. J. (1992) *Regul. Pept.* **42,** 111–122.
9. Blackburn, M. B., Kingan, T. G., Bodnar, W., Shabanowitz, J., Hunt, D. F., Kempe, T., Wagner, R. M., Raina, A. K., Schnee, M. E. & Ma, M. C. (1991) *Biochem. Biophys. Res. Commun.* **181,** 927–932.
10. Clottens, F. L., Holman, G. M., Coast, G. M., Totty, N. F., Hayes, T. K., Kay, I., Mallet, A. I., Wright, M. S., Chung, J.-S., Truong, O. & Bull, D. L. (1994) *Peptides* **15,** 971–979.
11. Furuya, K., Schegg, K. M., Wang, H., King, D. S. & Schooley, D. A. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 12323–12327.
12. Furuya, K., Schegg, K. M. & Schooley, D. A. (1998) *Peptides* **19,** 619–626.
13. Clark, T. M., Hayes, T. K. & Beyenbach, K. W. (1998) *Am. J. Physiol. Renal Physiol.* **274,** F834–F840.
14. Furuya, K., Harper, M., Schegg, K. M. & Schooley, D. A. (2000) *Insect Biochem. Mol. Biol.* **30,** 127–133.
15. Lovejoy, D. A. & Balment, R. J. (1999) *Gen. Comp. Endocrinol.* **115,** 1–22.
16. Spina, M., Merlo-Pich, E., Chan, R. K. W., Basso, A. M., Rivier, J., Vale, W. & Koob, G. F. (1996) *Science* **273,** 1561–1564.
17. Moreau, J. L., Kilpatrick, G. & Jenck, F. (1997) *Neuroreport* **8,** 1697–1701.
18. Szibbo, C. M. & Tobe, S. S. (1983) *Gen. Comp. Endocrinol.* **49,** 437–445.
19. Yamamoto, R. T. (1969) *J. Econ. Entomol.* **62,** 1427–1431.
20. King, D. S. (1996) *ABRF News* **7** (4), 26–28.
21. Audsley, N., Coast, G. M. & Schooley, D. A. (1993) *J. Exp. Biol.* **178,** 231–243.
22. Ramsay, J. A. (1954) *J. Exp. Biol.* **31,** 104–113.
23. Coast, G. M., Rayne, R. C., Hayes, T. K., Mallet, A. I., Thompson, K. S. J. & Bacon, J. P. (1993) *J. Exp. Biol.* **175,** 1–14.
24. Coast, G. M. (1995) *Regul. Pept.* **57,** 283–296.
25. Patel, M., Hayes, T. K. & Coast, G. M. (1995) *J. Exp. Biol.* **198,** 793–804.
26. Maddrell, S. H. P. & O'Donnell, M. J. (1993) *J. Exp. Biol.* **177,** 287–292.
27. Orlowski, R. C., Epand, R. M. & Stafford, A. R. (1987) *Eur. J. Biochem.* **162,** 399–402.
28. Yates, A. J., Gutierrez, G. E., Garrett, I. R., Mencel, J. J., Nuss, G. W., Schreiber, A. B. & Mundy, G. R. (1990) *Endocrinology* **126,** 2845–2849.
29. Reagan, J. D. (1995) *Insect Biochem. Mol. Biol.* **25,** 535–539.
30. Copley, K. S., Welch, W. H. & Schooley, D. A. (1996) in *Protein Society 10th Symposium* (Cambridge Univ. Press, San Jose, CA), p. 99.
31. Reagan, J. D. (1994) *J. Biol. Chem.* **269,** 9–12.
32. Clark, T. M., Hayes, T. K., Holman, G. M. & Beyenbach, K. W. (1998) *J. Exp. Biol.* **201,** 1753–1762.
33. Gracia-Navarro, F., Lamacz, M., Tonon, M. C. & Vaudry, H. (1992) *Endocrinology* **131,** 1069–1074.
34. Tatsuno, I., Yada, T., Vigh, S., Hidaka, H. & Arimura, A. (1992) *Endocrinology* **131,** 73–81.
35. Yada, T., Vigh, S. & Arimura, A. (1993) *Peptides* **14,** 235–239.
36. Hurwitz, S. (1996) *Crit. Rev. Biochem. Mol. Biol.* **31,** 41–100.
37. Chakraborty, M., Chatterjee, D., Kellokumpu, S., Rasmussen, H. & Baron, R. (1991) *Science* **251,** 1078–1082.
38. Force, T., Bonventre, J. V., Flannery, M. R., Gorn, A. H., Yamin, M. & Goldring, S. R. (1992) *Am. J. Physiol.* **262,** F1110–F1115.
39. Chabre, O., Conklin, B. R., Lin, H. Y., Lodish, H. F., Wilson, E., Ives, H. E., Catanzariti, L., Hemmings, B. A. & Bourne, H. R. (1992) *Mol. Endocrinol.* **6,** 551–556.
40. Arrese, E. L., Flowers, M. T., Gazard, J. L. & Wells, M. A. (1999) *J. Lipid Res.* **40,** 556–564.
41. Thompson, J. D., Higgins, D. G. & Gibson, T. J. (1994) *Nucleic Acids Res.* **22,** 4673–4680.
42. Adams, M. D., Celniker, S. E., Holt, R. A., Evans, C. A., Gocayne, J. D., Amanatides, P. G., Scherer, S. E., Li, P. W., Hoskins, R. A., Galle, R. F., *et al.* (2000) *Science* **287,** 2185–2195.
43. Laenen, B. (1999) Ph.D. thesis (Limburgs Universitair Centrum, Belgium).