Amino acid sequence and biological activity of a calcitonin-like diuretic hormone (DH31) from Rhodnius prolixus

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SUMMARY
Diuresis in the blood-gorging hemipteran Rhodnius prolixus is under neurohormonal control and involves a variety of processes and tissues. These include ion and water movement across the epithelium of the crop and the Malpighian tubules, and muscle contractions of the crop, hindgut and dorsal vessel, which facilitate mixing of the blood-meal, mixing of the haemolymph, as well as the expulsion of waste. One of the neurohormones that might play a role in this rapid diuresis belongs to the calcitonin-like diuretic hormone (DH31) family of insect peptides. Previously we have demonstrated the presence of DH31-like peptides in the central nervous system (CNS) and gut of R. prolixus 5th instars. In the present work, a DH31 from the CNS of 5th instar R. prolixus was isolated using reversed-phase liquid chromatography (RPLC), monitored with an enzyme-linked immunosorbent assay (ELISA) combined with matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry, and sequenced using tandem mass spectrometry and Edman degradation. This neuropeptide is the first to be sequenced in R. prolixus and has a sequence identical to that found previously for Dippu-DH31 from the cockroach Diploptera punctata. In previous studies testing Rhopr/Dippu-DH31 in Malpighian tubule secretion assays, we demonstrated increases in the rate of secretion that were small, relative to that induced by serotonin, but nevertheless 14-fold over baseline. In the present study, we investigated second messenger pathways in response to Rhopr/Dippu-DH31 and found no increase or decrease in cyclic adenosine monophosphate (cyclic AMP) content of the Malpighian tubules. DH31-like immunoreactivity is present over the dorsal hindgut, anterior dorsal vessel and dorsal diaphragm, and bioassays of the R. prolixus dorsal vessel and hindgut indicate that Rhopr/Dippu-DH31 increases the frequency of muscle contractions of both tissues. Second messenger pathways were also investigated for the dorsal vessel and hindgut.

Key words: amino acid, Rhodnius prolixus, calcitonin, diuretic hormone.

INTRODUCTION
Rhodnius prolixus is a blood-feeding hemipteran and is one of the species responsible for the transmission of Chagas’ disease. Rhodnius ingest enormous blood-meals and then undergo a period of rapid diuresis for 2–3 h during which they excrete approximately 40% of the volume of the blood-meal. This rapid, post-feeding diuresis, is under neurohormonal control and involves the coordination and integration of several processes and tissues. These include ion and water movement across the epithelium of the crop and the Malpighian tubules, and muscle contractions of the crop, hindgut and dorsal vessel, which facilitate mixing of the blood-meal, mixing of the haemolymph, as well as the expulsion of waste. Several factors have been suggested to play a role during this rapid diuresis, including serotonin (Lange et al., 1988; Maddrell et al., 1991; Maddrell et al., 1993), corticotropin-releasing factor (CRF)-like diuretic hormone (DH) (Te Brugge et al., 1999) and kinin-like peptides (Te Brugge et al., 2001). These factors stimulate Malpighian tubule secretion and/or muscle contractions of the gut during diuresis, while the cardioactive peptide 2b (CAP2b)-like peptides decrease Malpighian tubule secretion in R. prolixus (Quinlan et al., 1997; Paluzzi and Orchard, 2006).

Another family of peptides, the calcitonin-like DH, were first isolated and sequenced from extracts of brain and corpus cardiacum (CC) of the cockroach D. punctata (Furuya et al., 2000), and shown to stimulate secretion of Malpighian tubules from both D. punctata and Locusta migratoria (Furuya et al., 2000). These DH31-like peptides have now been predicted from the genome or sequenced from tissue extracts of several species including Drosophila melanogaster (Coast et al., 2001), Anopheles gambiae (Coast et al., 2005), Aedes aegypti, Bombyx mori, Apis mellifera, Tribolium castaneum and Formica polyctena (Schooley et al., 2005). These sequences display a high degree of identity to the D. punctata sequence. The T. castaneum, B. mori, D. melanogaster and A. gambiae sequences have 94, 74, 71 and 68% identity, respectively, to the amino acid sequence from D. punctata (Table 1), while the sequences from A. mellifera and F. polyctena (with the exception of the last two unknown amino acids) are identical to the cockroach sequence (Table 1).

Using an affinity-purified antibody raised against Dippu-DH31 in whole-mount immunohistochemistry, we found Dippu-DH31-like immunoreactivity in cell bodies and processes throughout the central nervous system (CNS) of 5th instar R. prolixus (Te Brugge et al., 2005). Specifically, immunoreactivity was observed in the medial and lateral neurosecretory cells of the brain, which send processes to the retrocerebral complex, and in dorsal unpaired median (DUM) neurons of the mesothoracic ganglionic mass (MTGM), which send processes to neurohaemal sites on the surface of the abdominal nerves. Dippu-DH31-like immunoreactive processes were also observed over the anterior dorsal vessel, dorsal hindgut and the salivary glands. Immunohistochemical analysis 1 h
of Malpighian tubule secretion is not the main role for a DH31-like peptide for its possible activation of cyclic AMP pathways in insects, such as modifying the ionic composition of the urine, gut contraction or heart contraction. The presence of Dippu-DH31-like peptides on R. prolixus hindgut as well as other peripheral tissues, and that it plays other roles during diuresis, suggesting that the DH31-like material may be released during the rapid phase of diuresis (Te Brugge et al., 2005).

When tested on R. prolixus Malpighian tubules, Dippu-DH31 causes only small (relative to serotonin) but significant increases (14-fold) in the rate of secretion (Te Brugge et al., 2005). In other insects, such as D. punctata, L. migratoria and D. melanogaster, DH31 peptides stimulate larger increases in the rates of secretion (Furuya et al., 2000; Coast et al., 2001). In some insects this increase in secretion by DH31-like peptides is through a cyclic AMP-dependent pathway (Coast et al., 2001; Coast et al., 2005). It is possible that the native R. prolixus DH31 is sufficiently different from the other peptides such that the non-native peptides could be relatively inactive in R. prolixus. It is also possible that stimulation of Malpighian tubule secretion is not the main role for a DH31-like peptide in R. prolixus, and that it plays other roles during diuresis, such as modifying the ionic composition of the urine, gut contraction or heart contraction. The presence of Dippu-DH31-like immunoreactivity on the hindgut and the anterior dorsal vessel and its potential release as a neurohormone suggest a role for DH31-like peptides on R. prolixus hindgut as well as other peripheral tissues, such as the dorsal vessel and heart.

In order to further our understanding of the overall feeding-related neuroendocrinological events in Rhodnius, and to appreciate the cocktail of hormones (neuropeptides and amines) involved, we have quantified, analysed the distribution of, isolated and sequenced a native DH31, Rhopr/Dippu-DH31. We have tested this peptide for its possible activation of cyclic AMP pathways in Malpighian tubules, and also shown that Rhopr/Dippu-DH31 has potential roles in other tissues involved in rapid post-feeding diuresis.

**MATERIALS AND METHODS**

**Animals**

Fifth instars of R. prolixus (Stål 1859) were taken from a long-standing colony maintained at 25°C under 60% humidity. The insects were unfed, 6–8 weeks post-emergence, and previously fed on rabbit’s blood as 4th instars.

**Tissue collection**

CNS, whole or parts, from 5th instar male and female R. prolixus were dissected under physiological saline (Lane et al., 1975). Tissues were extracted in 1 ml of ice-cold acidified methanol (methanol:acetic acid:water; 90:9:1). The tissues in acidified methanol were frozen at –20°C, and later thawed, sonicated and centrifuged at 8800 g for 10 min. The supernatant was decanted and dried in a SpeedVac (Savant, Farmingdale, NY, USA) and frozen at –20°C until use.

**DH31 enzyme-linked immunosorbant assay**

The competitive DH31 enzyme-linked immunosorbant assay (ELISA) employed the same affinity-purified antibody as was used for immunohistochemistry and the direct ELISA described previously (Te Brugge et al., 2005). In brief, the procedure used 100 μl of primary antibody solution (1:500 anti-[Cys-32]Dippu-DH31 in Hapes-buffered saline (HBS; 10 mmol·l–1 Hapes, 150 mmol·l–1 NaCl, 1 mmol·l–1 MgCl2, pH 7.4), which was added to each well of a 96-well ELISA plate (Corning easy wash®, Corning, NY, USA). The plate was covered and incubated overnight at 4°C. The antibody was then discarded and the plate washed three times with 200 μl per well of washing buffer (0.05% Tween 20 in HBS, pH 7.4). The contents of the plate were then discarded and the plate blocked by adding 100 μl of 5% normal goat serum in HBS to each well and incubating for 1 h at room temperature on a flatbed shaker. The contents were again discarded and the plate blotted. Blank, zero, standards (5 to 10 000 fmol 50 μl–1 Dippu-DH31), samples and alkaline phosphatase [Cys-32]Dippu-DH31 conjugate (1:2000) were made up in HBS with 0.1% bovine serum albumin (BSA). Standards and samples, run in duplicate, were added in a volume of 50 μl followed by the addition of an equal volume of the DH31 alkaline phosphatase conjugate to each well. The plate was covered and incubated at room temperature for 3 h on the flatbed shaker. Subsequently, the contents of the plate were discarded and the plate was washed three times with 200 μl of washing buffer and then blotted. To each well, 100 μl of p-nitrophenyl phosphate (pNPP) liquid substrate system (Sigma-Aldrich, St Louis, MO, USA) was added, and the plate covered and then placed on a flatbed shaker for 1.5 h at room temperature. The plate was read in a Molecular Devices Spectramax microplate reader at 405 nm (Molecular Devices, Sunnyvale, CA, USA).

**Tissue collection for DH31 purification**

Four-hundred 5th instar R. prolixus CNS were dissected under physiological saline. The tissues were extracted in batches of 50 in 1 ml of ice-cold acidified methanol. The tissues in acidified methanol were frozen at –20°C, then later thawed, sonicated and centrifuged at 8800 g (10 000 r.p.m.) for 10 min. The supernatant was decanted and dried in a SpeedVac. These tissue extracts were brought up in 0.1% trifluoroacetic acid (TFA; BDH, Toronto, ON, Canada) in water and applied to a C18 Sep-Pak cartridge (Waters Associates, Mississauga, ON, Canada) that had previously been equilibrated with...
sequential applications of 8 ml methanol, then 8 ml water, then 8 ml water containing 0.1% TFA, and finally 2 ml of 0.1% TFA in water with 10 μg of protease-free BSA (Sigma-Aldrich). The cartridge was then washed sequentially with 8.0 ml each of water with 0.1% TFA then 99.9% methanol (Burrock and Jackson, Muskegon, MI, USA) with 0.1% TFA, and the eluents collected. We chose to use 100% methanol in order to preserve all of the possible neuropeptide families, since our interests extend beyond DH31 alone. The collected extracts were dried in the SpeedVac and frozen at –20°C until use.

**Reversed-phase high pressure liquid chromatography (RPLC) purification**

The dried Sep-Pak eluents were further purified by RPLC in two batches containing 150 and 250 CNS equivalents. Two RPLC purification steps were used and are described below.

**System A**

The dried eluents were brought into solution in 9% acetonitrile (Burrock and Jackson) with 0.1% TFA and filtered using a 0.22 μm Spin-X® filter (Corning). The filtrate was then applied to an RPLC system that utilised a Brownlee C18 column (Mandel/Alltech, Guelph, ON, Canada). The RPLC gradient was from 9% to 60% acetonitrile with 0.1% TFA over 34 min (1.5% acetonitrile per min) at a flow rate of 1 ml min⁻¹. Fractions were collected, aliquoted, dried and stored at –20°C until use.

**System B**

Active fractions, as determined by DH31 ELISA, were further purified through an RPLC system that utilised a Brownlee Spheri 5 phenyl column (Mandel/Alltech). The fractions were brought into solution in 1 ml of 18% acetonitrile with 0.1% TFA. The RPLC gradient was from 18% to 60% acetonitrile over 60 min (0.7% acetonitrile per min) collected in 1 ml fractions. The fractions were aliquoted, dried and stored at –20°C until use.

**Mass spectrometry and Edman degradation sequencing**

The isolated sample with DH31-like immunoreactivity from 150 CNS was dried by SpeedVac and analysed by the Centre of Advanced Protein Technology (Hospital for Sick Children, Toronto, ON, Canada). Samples were analysed using matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectrometry (Q-Star, Applied Biosystems Inc. Sciex, Concord, ON, Canada). Peaks observed in the MALDI-TOF analysis of specific molecular weights were subjected to further analysis using tandem mass spectrometry (MALDI-TOF MS/MS) and trypsin digestion.

The second batch of 250 CNS were dried and processed on RPLC in a similar manner, with the DH31-like immunoreactive fraction further subjected to molecular mass determination and sequencing. This sample was dissolved in 1% aqueous TFA with sonication and then injected onto a 3.5 μm Zorbax 300SB C8 column, 2.1 mm × 100 mm, using a 5 ml loop (Rheodyne Model 7125, Rohnert Park, CA, USA) fitted to a Hewlett Packard Model 1050 chromatograph. The sample was eluted with a gradient starting at 5% acetonitrile/0.1% TFA, increasing over 5 min to 20% acetonitrile/0.1% TFA, then increasing to 45% acetonitrile/0.1% TFA at 65 min. Peaks were collected based on their absorbance at 280 nm, and aliquots analysed by MALDI-TOF MS using an Applied Biosystems Model 4700 spectrometer (Framingham, MA, USA). Aliquots of peaks of interest were then digested with trypsin and fragments were analysed by tandem MS (Applied Biosystems Model 4700 spectrometer). Approximately half of the intact peptide was submitted for sequence analysis by Edman degradation using an Applied Biosystems Prociase Model 494 sequencer (Foster City, CA, USA).

**Cyclic AMP assays**

The cyclic AMP content of the R. prolixus 5th instar Malpighian tubules, hindgut and dorsal vessel was assayed either with or without 3-isobutyl-1-methylxanthine (IBMX). Tissues from 5th instars were dissected and then transferred to a microfuge tube. The incubations with IBMX (0.5 mmol l⁻¹) were conducted in a total volume of 50 μl in physiological saline, or physiological saline containing serotonin (10⁻⁶ mol l⁻¹) or Rhopr/Dippu-DH31 (10⁻⁸ mol l⁻¹, 10⁻⁶ mol l⁻¹). Tissues were incubated for 10 min at room temperature and then the reaction was stopped with 500 μl of boilling 0.05 mol l⁻¹ sodium acetate (pH 6.2). In assays where no IBMX was used the tissues were incubated in a volume of 50 μl at room temperature for either 1 or 10 min. The assays were then stopped with 250 μl of boiling sodium acetate. After the addition of the sodium acetate, samples were placed in a boiling water bath for a further 5 min, then frozen at –20°C until assayed. The samples were thawed, sonicated and centrifuged at 8800 g for 10 min and the supernatant decanted.

The cyclic AMP content of the supernatant was measured using an RIA kit (Perkin Elmer, Woodbridge, ON, Canada) with
modifications as previously described (Lange and Orchard, 1986). The means ± s.e.m. were calculated and compared using Student’s unpaired t-test (N=5–10).

**DH31-like immunoreactivity of the R. prolixus 5th instar dorsal diaphragm and heart**

Fifth instar *R. prolixus* were placed dorsal surface down into paraffin wax and dissected under physiological saline from the ventral surface. Cuticle from ventral abdominal segments 1–7 was removed, as was the ventral diaphragm, gut and Malpighian tubules. This exposed the dorsal vessel and heart without damaging the alary muscles or the dorsal diaphragm. Dorsal abdominal segments posterior to segment 5 were transferred to a solution of 2% paraformaldehyde in Millonig’s buffer. Immunohistochemistry was performed as described previously (Tsang and Orchard, 1991; Te Brugge et al., 2005), with only minor modifications. The primary antiseraum solution utilised the affinity-purified rabbit anti-DH31 at 1:500 in 0.4% Triton X-100 with 10% normal sheep serum and incubation was carried out at 4°C for 48 h. Secondary antibody was Cy3-labelled sheep anti-rabbit immunoglobulin at 1:200 dilution (Sigma-Aldrich). In order to show more clearly the alary muscles, heart and dorsal vessel, some fixed preparations were exposed to a solution containing phalloidin conjugated to TRITC (Sigma-Aldrich) at a concentration of 1:1000 in PBS for 0.5 h at room temperature, followed by a wash in PBS for 18 h at 4°C. All preparations were mounted on slides in glycerol and viewed with a confocal microscope (Zeiss, LSM 510).

**Heart assay**

Fifth instar *R. prolixus* were placed dorsal surface down into paraffin wax and dissected under physiological saline from the ventral surface, as described above. Segments 5–10 of the dorsal cuticle were removed and transferred to a Sylgard (Paisley Products, Scarborough, ON, Canada)-coated dish, covered with 100 µl of physiological saline and secured by minutin pins. Electrodes from an impedance converter (UFI model 2991, Morro Bay, CA, USA) were placed on either side of the anterior-most pair of the heart’s alary muscles. Contractions of the heart, detected by the impedance monitor (set on alternating current, AC, short), were recorded on a chart recorder (Linear 1200; VWR, Mississauga, ON, Canada). The tissue was equilibrated in 100 µl of saline for 20 min at room temperature, during which the surrounding fluid was replaced with fresh saline every 5 min. After this period, the saline was removed and replaced with an equal volume of either saline or test solutions. The Rhopr/Dippu-DH31 was tested at concentrations ranging from $10^{-12}$ to $10^{-6}$ mol l$^{-1}$ (N=5–9).

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**Fig. 2.** Purification of DH31-like material from 150 *R. prolixus* CNS through RPLC system A. The active fraction 28 was then run through system B. Aliquots were tested by a DH31 ELISA to determine DH31-like immunoreactive fractions. The elution times of Drome-DH31 and Dippu-DH31 are indicated by the asterisks.

**Fig. 3.** MALDI-TOF (matrix-assisted laser desorption/ionisation time-of-flight) mass spectrometry of DH31-like material from phenyl column (system B) fraction 40. Peak at 2986.6 matches the MH$^+$ determined for Dippu-DH31. $M$, mass number; $Z$, atomic number.
Hindgut assay

Hindgut assays were conducted on isolated *R. prolixus* 5th instar hindguts maintained under physiological saline. The hindgut was dissected along with a portion of the ventral cuticle from abdominal segments 5–10 including the cuticle surrounding the anus. The ventral cuticle was used to secure the hindgut to a dish, coated with Sylgard. The anterior end of the hindgut, at the junction with the posterior midgut, was tied by a fine silk thread to a miniature force transducer (Aksjeselskapet Mikro-elektronikk, Horten, Norway). The output from the transducer was connected to a data acquisition system (Biopac MP 100 workstation, Biopac Systems Inc., Santa Barbara, CA, USA) for measuring changes in basal tosun as well as phasic contractions. Tissues were equilibrated in 50·mol·l−1 IBMX, isobutyl methylxanthine. *Significantly different from saline control values (Student’s unpaired t-test, P<0.05).

### RESULTS

#### Quantification of DH31-like material in 5th instar CNS

The DH31 ELISA is a competitive ELISA that is sensitive (able to detect) over a range of 25 to 1000·pmol Dippu-DH31. The same antibody has been tested and shown to be specific for DH 31-like peptides with some cross-reactivity with vertebrate calcitonin-like peptides (Te Brugge et al., 2005). Dilutions of the *R. prolixus* CNS extracts paralleled the Dippu-DH31 standard curve (Fig. 1A). Fifth instar *R. prolixus* CNS contained 1.2±0.17·pmol of DH31-like material. There was no difference found in the amount of DH31-like material in the CNS of male and female 5th instar *R. prolixus* (data not shown). The brain and MTGM contained the majority of the Dippu-DH31-like activity (595±45 and 325±54·pmol, respectively; Fig. 1B). Smaller amounts of DH31-like material were measured in the suboesophageal ganglion (SOG) and prothoracic ganglion (PRO) and in the neurohaemal areas of the CC and abdominal nerves (ABN).

#### RPLC purification of *R. prolixus* DH31-like material

DH31-like material extracted from 150 *R. prolixus* CNS and separated by using RPLC over a C18 column (system A) eluted in two major peaks of activity; one in fractions 27, 28 and 29 (40.5–45% acetonitrile) and a second much smaller peak in 21% acetonitrile. The estimated amount of DH31-like activity in fraction 40, using the DH31 ELISA, was approximately 34.6·pmol. The DH31 immunoreactive material purified from the batch of 250 CNS eluted at a similar time and percentage acetonitrile to the batch of 150 CNS in both the C18 and phenyl RPLC runs. An estimated 85.6·pmol of DH31-like material was found in fraction 40 from this run. Known DH31-like peptides were run on system B after the *R. prolixus* samples. The *D. melanogaster* (Drome-DH31) standard eluted from the column at 37.9·min (fraction 38) while the Dippu-DH31 standard eluted at 39.3·min (fraction 40).

#### DH31 analysis: MALDI-TOF MS, MALDI-TOF MS/MS and Edman degradation

An aliquot of fraction 40 from the phenyl RPLC run of 150 CNS was analysed by MALDI-TOF MS and shown to contain a mass corresponding to the mass of Dippu-DH31. This peak was further analysed by MALDI-TOF MS/MS and trypsin digestion; the results were consistent with a peptide identical to Dippu-DH31 except for the inability to distinguish between leucine and isoleucine.

Fraction 40 from the second batch of 250 CNS tissues was further separated by RPLC using a Zorbax C8 column and peaks were collected based on their absorbance at 280·nm and aliquots analysed by MALDI-TOF MS. A peak eluting at 29.86.61·min had MH+ 2986.61·pmol of DH31-like material was found in fraction 28, was dried in a SpeedVac and run again on RPLC using a phenyl column (system B). These fractions were collected, and aliquots tested in the DH31 ELISA. DH31-like material eluted from the phenyl column in fraction 40 (approximately 42% acetonitrile; Fig. 2B). The estimated amount of DH31-like activity in fraction 40, using the DH31 ELISA, was approximately 34.6·pmol. The DH31 immunoreactive material purified from the batch of 250 CNS eluted at a similar time and percentage acetonitrile to the batch of 150 CNS in both the C18 and phenyl RPLC runs. An estimated 85.6·pmol of DH31-like material was found in fraction 40 from this run. Known DH31-like peptides were run on system B after the *R. prolixus* samples. The *D. melanogaster* (Drome-DH31) standard eluted from the column at 37.9·min (fraction 38) while the Dippu-DH31 standard eluted at 39.3·min (fraction 40).

### Cyclic AMP assays

The cyclic AMP content of the Malpighian tubules incubated with saline, Rhopr/Dippu-DH31 or serotonin was measured in the presence or absence of IBMX. There was no significant increase in the cyclic AMP content of the Malpighian tubules incubated with 10−6 or 10−8·mol·l−1 Rhopr/Dippu-DH31 for 1·min, 10·min or 10·min in IBMX, whereas 10−6·mol·l−1 serotonin significantly increased cyclic AMP under each of these conditions.
Both Rhopr/Dippu-DH31 and serotonin did, however, increase the cyclic AMP content of dorsal vessel and of hindgut, when incubated for 10 min in the presence of IBMX (Table 3).

**DH31-like immunoreactivity of the heart and alary muscles**

The *R. prolixus* dorsal vessel and heart are located within the dorsal diaphragm. The heart and alary muscles are confined to the posterior portion of the abdomen in segments 5–10 (Fig. 4A). Phalloidin staining of the dorsal diaphragm outlines the structure of the heart and alary muscles (Fig. 4B). The alary muscles are attached to both the ventral and dorsal cuticle in these segments.

The dorsal vessel (aorta) at the CC continues anteriorly towards the head forming a hood-like structure over the CC. DH31-like immunoreactivity was seen over the anterior portion of the dorsal vessel, at the CC, and continuing along the dorsal vessel to the posterior end of the thorax (not shown). No DH31-like immunoreactivity was seen directly innervating the heart, alary muscles or posterior dorsal vessel; however, there was DH31-like immunoreactivity in processes, derived from the abdominal nerves, over the dorsal diaphragm (Fig. 4C) that come in close proximity to the alary muscles and heart.

**Heart bioassay**

Contractions recorded with the impedance monitor were seen to start with a contraction of the alary muscles, followed by the relaxation of the alary muscles combined with the contraction of the heart, and finally the contraction of the dorsal vessel (Fig. 5). The majority of the contractions under our experimental conditions were anterograde, with no consistent pattern of retrograde contractions observed in either saline or saline containing Rhopr/Dippu-DH31. The mean frequency of contractions in saline was 5.6±1.2 contractions min⁻¹ (N=7). Addition of Rhopr/Dippu-DH31 increased the frequency of contractions over the saline controls in a dose-dependent manner (Figs 6 and 7), with a threshold (lowest concentration to produce a significant increase in frequency) of approximately 10⁻¹¹ mol l⁻¹.

**Hindgut bioassay**

Contractions of the *R. prolixus* hindgut are composed of contractions of circular and longitudinal muscles, resulting in a complex and variable pattern of contraction (Fig. 8). The frequency of contractions in *R. prolixus* saline was 2.3±0.3 contractions min⁻¹.
The variability appeared, in part, to be dependent on the feeding state of the bug and the amount of residual material in the hindgut. Rhopr/Dippu-DH31 increased the frequency of contractions in a dose-dependent manner (Figs 8 and 9) with a threshold of 10^{-11} mol l^{-1}.

**DISCUSSION**

We have isolated and sequenced a calcitonin-like DH31 peptide from the CNS of 5th instar *R. prolixus*. This is the first neuropeptide to be sequenced from the CNS of *R. prolixus*. The peptide is identical to Dippu-DH31 sequenced from the cockroach *D. punctata* (Furuya et al., 2000), and is therefore called Rhopr/Dippu-DH31. Identical sequences have now been isolated and sequenced (*D. punctata* and *R. prolixus*) or predicted from genomes (bee), while other insect DH31-like peptides have a high degree of identity in their amino acid sequences (Table 1). These results suggest that the DH31 peptides are highly conserved across orders of insects as well as between insects utilising various feeding strategies, suggesting that this family of peptides plays an important role in diuresis and possibly feeding. In species of insects other than *R. prolixus*, only one form of DH31 peptide has been detected. However, it is interesting that in *R. prolixus* a second, smaller peak of DH31-like immunoreactive material elutes later in the RPLC purification. Whether this represents a second DH31-like peptide or represents cross-reactivity with another peptide must await further study.

Quantification of the DH31-like material in the *R. prolixus* CNS demonstrated approximately 1.2 pmol per CNS of DH31-like immunoreactive material. The distribution of DH31-like immunoreactive material is consistent with the immunohistochemical staining of CNS neurons and processes, with the number of neurons highest in the brain and MTGM, and smaller amounts of DH31-like material in the putative neurohaemal sites of the CC and ABN. No other studies have quantified DH31 in insect CNS and so no comparisons can be made.

DH31-like immunoreactivity is co-localised with serotonin in the DUM cells of the MTGM and in the associated neurohaemal terminals of the abdominal nerves (Te Brugge et al., 2005). Serotonin is a true diuretic hormone in *R. prolixus* (Maddrell et al., 1991), released from these neurohaemal terminals in response to feeding, with its titre increasing in the haemolymph during the first 5 min of feeding (Lange et al., 1989). Thus, it is intriguing that DH31-like immunoreactivity is co-localised with serotonin in these DUM cells and terminals as it suggests that Rhopr-DH31 is probably co-released with serotonin at the start of feeding (Te Brugge et al., 2005), and that it is a neurohormone associated with feeding. However, there are other neurons in the CNS that express DH31-like immunoreactivity but do not co-express serotonin. It is therefore possible that Rhopr/Dippu-DH31 can be released on its own, or in combination with serotonin, suggesting that fine levels of control are possible.

Previously we have tested Dippu-DH31 on *R. prolixus* Malpighian tubules (Te Brugge et al., 2005). Since the sequence of the *R. prolixus* peptide is identical to that of the cockroach peptide we can...
Interestingly, Rhopr/Dippu-DH31 stimulates an increase in amplitude. The second messenger pathway for DH31-like peptides has been now confirmed that the native R. prolixus DH31 peptide does not stimulate the high rates of secretion (1000-fold) seen for serotonin on R. prolixus Malpighian tubules. However, Rhopr/Dippu-DH31 does stimulate a 14-fold increase in secretion. It may, of course, also play other roles in Malpighian tubules, such as modifying the ionic composition of the primary urine, but this is yet to be tested.

The second messenger pathway for DH31-like peptides has been studied in the Malpighian tubules of several species. In both D. melanogaster (Coast et al., 2001) and A. gambiae (Coast et al., 2005) the native DH31-like peptide increases the production of cyclic AMP, while cyclic GMP production is unchanged in the presence of IBMX. Dippu-DH31 increases the cyclic AMP content of Schistocerca americana tubules, but not tubules of Manduca sexta (Furuya et al., 2000) or of D. punctata (Tobe et al., 2005). Interestingly, Rhopr/Dippu-DH31 does not elevate the cyclic AMP content of R. prolixus tubules, despite being capable of increasing secretion 14-fold. In this manner, R. prolixus tubules act more like M. sexta and D. punctata tubules.

In previous studies DH31-like immunoreactivity was observed on the anterior dorsal vessel and the hindgut suggesting that Rhopr/Dippu-DH31 could be acting directly on these tissues, and/or released from these terminals into the haemolymph (Te Brugge et al., 2005). Interestingly, Rhopr/Dippu-DH31 stimulates an increase in the cyclic AMP content of the dorsal vessel and the hindgut but does not increase the cyclic AMP content of the Malpighian tubules of R. prolixus. This difference, in R. prolixus, between the Malpighian tubules, dorsal vessel and hindgut in the production of cyclic AMP suggests the possibility of a difference in the Rhopr/Dippu-DH31 receptors on the tubules versus the dorsal vessel and hindgut, or possibly a difference in the G-proteins that couple to the receptor for DH31.

DH31-like immunoreactivity was observed on the anterior portion of the dorsal vessel, in processes that are putative neurohaemal release sites and in processes that extend over the dorsal diaphragm close to the alary muscles, heart and dorsal vessel. No direct innervation of the alary muscles or heart was observed. The heart and dorsal vessel play an important role in moving haemolymph from the posterior to the anterior (or occasionally the reverse) of the bug. This is potentially all the more important after ingestion of the large blood-meal, since the expanded crop may confine haemolymph to anterior or posterior parts of the engorged bug. The dorsal vessel is capable of producing rates of flow approaching 4 μl min⁻¹ (Amaka Enh, personal communication) and so may play a significant role in the circulation of haemolymph and hormones. Previous studies have examined the contraction of the heart and dorsal vessel of R. prolixus (Chiang et al., 1992; Sarkar et al., 2003). Interestingly, both serotonin (Chiang et al., 1992) and Rhopr/Dippu-DH31 (this study) increase the frequency of contractions in a dose-dependent manner, with cyclic AMP possibly acting as the second messenger.

DH31-like immunoreactivity is also present over the dorsal hindgut and this immunoreactivity is diminished in intensity 1 h after feeding, suggesting that the DH31-like material in these terminals had been released (Te Brugge et al., 2005). The hindgut of R. prolixus plays an integral role during rapid post-feeding diuresis. The Malpighian tubules empty their contents into the hindgut through the ampullae. Contractions of the hindgut are then necessary to expel the hindgut contents and are also potentially important for mixing of both the hindgut contents and the surrounding haemolymph. During rapid post-feeding diuresis the hindgut expels its contents every few minutes. Since the hindgut of R. prolixus is where the mature Trypanosome parasites reside prior to being expelled following diuresis/excretion, it is of some considerable interest to understand the factors that might affect this
Dippu-DH46 is a potent synergist of Dippu-DH31 (Furuya et al., 2005) which both increase basal tonus, and increase the frequency of hindgut contractions, but did not cause a change in basal tonus. Again, in contrast to Malpighian tubules, but in a manner similar to dorsal vessel, Rhopr/Dippu-DH31 might act via cyclic AMP as a second messenger in hindgut muscle. These results, taken together, suggest that Rhopr/Dippu-DH31 may be playing an active role in modulating the hindgut during the rapid post-feeding diuresis/excretion in R. prolixus.

The DH31 family of peptides in insects is highly conserved and is involved in ion and water balance as well as muscle contraction. The native R. prolixus DH31, Rhopr/Dippu-DH31, would appear to play an integral role in this diuresis, stimulating low levels of secretion by the Malpighian tubules, and stimulating contractions of both the heart and hindgut (tissues that are associated with the diuretic behaviour). The interaction of Rhopr/Dippu-DH31 with other factors, in particular serotonin, and its role in Malpighian tubules and the digestive system will be of importance for future study; Dippu-DH31 is a potent synergist of the action of the CRF-like DH Dippu-DH46 in Diploptera punctata, and conversely Dippu-DH46 is a potent synergist of Dippu-DH31 (Furuya et al., 2000). Diuresis in R. prolixus appears to involve complex and tightly integrated events that incorporate a variety of tissues that are under neurohormonal/neuromodulatory control of both amines and neuropeptides.

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