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Distribution, activity and evidence for the release of an anti-diuretic peptide in the kissing bug *Rhodnius prolixus*

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Summary

In the haematophagous insect *Rhodnius prolixus*, diuresis is accomplished through the combined actions of peptidergic diuretic hormones and 5-HT released from neurohaemal sites on the abdominal nerves. Preliminary work on anti-diuresis in this blood-feeder, previously believed to occur through a decrease in the levels of the diuretic factors, indicates that an anti-diuretic hormone, with properties similar to CAP2b (pELYAFPRVamide; recently renamed Mas-CAPA-1), might also be present in *R. prolixus*. Here, we present evidence from immunohistochemical analysis that suggests a PRXamide-like neuropeptide may be released from the abdominal neurohaemal sites beginning 3–4 h following feeding; a time that coincides with the cessation of diuresis. We also show evidence for an endogenous factor, isolated from the central nervous system using reversed-phase high performance liquid chromatography, which mimics the effects of Mas-CAPA-1. Specifically, this endogenous anti-diuretic factor inhibits rates of 5-HT-stimulated secretion in a dose-dependent manner and elevates intracellular cGMP levels of Malpighian tubules stimulated with 5-HT.

Key words: anti-diuresis, CAPA, CAP2b, neuropeptide, neurosecretory cells, immunohistochemistry, insect, Malpighian tubule, *Rhodnius prolixus*.

Introduction

In the haematophagous insect *Rhodnius prolixus* tubule fluid secretion is accomplished through the combined actions of peptidergic diuretic hormones and 5-hydroxytryptamine (5-HT) (see Maddrell et al., 1971; Maddrell et al., 1993; Te Brugge et al., 1999; Te Brugge et al., 2005) acting through the intracellular second messenger, cyclic 3′,5′-adenosine monophosphate (cAMP) (see Barrett and Orchard, 1990; Montoreano et al., 1990; Te Brugge et al., 1999). Similar mechanisms controlling diuresis exist in other insect species (see Coast et al., 2002) but, in addition, in *Drosophila melanogaster* and *Manduca sexta*, another second messenger, cyclic 3′,5′-guanosine monophosphate (cGMP) has also been shown to be involved in increasing the rate of tubule fluid secretion (Skaer et al., 2002; Davies et al., 1995).

Anti-diuresis in *R. prolixus*, or the cessation of diuresis, has typically been considered to occur through a decrease in the levels of diuretic hormones 3–4 h following feeding (Maddrell, 1964). More recent studies on *R. prolixus* have identified MasCAP2b (*M. sexta* cardioactive peptide 2b) and cGMP as components of an anti-diuretic mechanism (Quinlan et al., 1997). Specifically, cGMP was identified as an intracellular second messenger to MasCAP2b, and Malpighian tubule cGMP levels were shown to increase in response to MasCAP2b and also as tubule secretion rates declined *in vivo* (Quinlan et al., 1997). In addition, application of cGMP to tubules elicited effects that were antagonistic to the secretory effects of cAMP (Quinlan and O'Donnell, 1998). It has been proposed that cGMP activates a cAMP phosphodiesterase that degrades cAMP, thus lowering the level of the second messenger that stimulates diuresis (O'Donnell and Spring, 2000).

MasCAP2b (pELYAFPRVamide, recently renamed Mas-CAPA-1, see later) is a cardioactive peptide first isolated in *M. sexta* (Huesmann et al., 1995). It is now known that MasCAP2b is a member of a family of peptides sharing the C-terminal PRVamide motif (Loi and Tublitz, 2004). In the central nervous system (CNS) these include some periviscerokinins (see Wegener et al., 2002) and CAP2b-related peptides in *D. melanogaster* and *M. sexta* (Kean et al., 2002; Loi and Tublitz, 2004). In the periphery, the PRVamide motif is retained by *M. sexta* pre-ecdysis-triggering hormone (MasPETH) from the peripheral endocrine Inka cells. Some other related peptides have a C-terminal PRXamide motif (where X=I, L, M or V). For example, in Lepidopteran species, these include the pheromone biosynthesis activating neuropeptides (PBAN) (see Teal et al., 1996) within the central nervous system, and peripherally include ecdysis-triggering hormone (ETH) (Žitnaň et al., 2002).

Recent studies have isolated and sequenced the gene coding for MasCAP2b (Loi and Tublitz, 2004). Owing to its high degree of homology with the *capability* gene in *D. melanogaster* (Kean et al., 2002), it was named the *Manduca* CAPA gene (Loi and Tublitz, 2004). This gene encodes three propeptides, a CAP2b propeptide and two CAP2b-related propeptides referred to as Mas-CAPA-1, Mas-CAPA-2 and Mas-pyrokinin-1 (Mas-PK-1), respectively (Loi and Tublitz, 2004). The *capability* gene in *D. melanogaster* encodes three neuropeptides termed CAPA-1 and CAPA-2, which are CAP2b related, while CAPA-3 is PBAN/PK related (Kean et al., 2002). To avoid confusion, we will follow the more recent nomenclature and subsequently refer to MasCAP2b as Mas-CAPA-1.

Given the recent finding suggesting a novel anti-diuretic mechanism in *R. prolixus* involving a Mas-CAPA-1-like peptide and the intracellular second messenger, cyclic GMP (Quinlan et al., 1997), we sought to map the location of putative Mas-CAPA-1-like immunoreactive cells and to seek evidence for an endogenous Mas-CAPA-1-like neuropeptide in *R. prolixus* with anti-diuretic properties. Here we describe the distribution of PRXamide-like immunoreactive neurons and neurohaemal sites in *R. prolixus* using an antiserum against MasPETH that recognizes Mas-CAPA-1. In addition, we provide evidence for the presence of an endogenous Mas-CAPA-1-like factor from the central nervous system (CNS) of *R. prolixus* that inhibits 5-HT-stimulated diuresis and elevates cGMP levels in 5-HT-stimulated tubules.

Materials and methods

Animals

Fifth-instar *Rhodnius prolixus* Stål were reared at high relative humidity in incubators at 25°C and routinely fed on rabbits' blood. Experiments were conducted on tissues of the CNS in both unfed animals (approximately 6 weeks post-ecdysis) and recently fed animals of both sexes.

Immunohistochemical staining

The insects were pinned ventral surface down, and the dorsal cuticle, dorsal diaphragm and digestive tissue removed under physiological saline (NaCl, 150 mmol l^{-1}; KCl, 8.6 mmol l^{-1}; $CaCl_2$, 2 mmol l^{-1}; $NaHCO_3$, 4 mmol l^{-1}; glucose, 34 mmol l^{-1}; $MgCl_2$, 8.5 mmol l^{-1}; Hepes pH 7.0, 5 mmol l^{-1}). The nervous tissue was fixed *in situ* with 2% paraformaldehyde (pH 7.0) at 4°C overnight (16–18 h). Following fixation, the tissues were washed and the CNS and short stretches of peripheral nerves removed under phosphate-buffered saline (PBS) (Lange et al., 1988). The nervous tissue was incubated in 4% Triton X-100, 2% bovine serum albumin (BSA) and 10% normal sheep serum (NSS) in PBS for 1 h at room temperature followed by several washings with PBS. The polyclonal rabbit antiserum to MasPETH (generously provided by Dr Dusan Žitnaň and Dr Mike Adams) diluted 1:1000 was preincubated in a 0.4% Triton X-100, 2% bovine serum albumin (BSA) and 2% normal sheep serum (NSS) in PBS at 4°C overnight (16–18 h) prior to use. The nervous tissue was then incubated in the antiserum for 48 h on a flatbed shaker at 4°C. Following this, tissues were washed several times in PBS, including an overnight washing at 4°C with shaking. Tissues were subsequently incubated overnight (16–18 h) with Cy3-labelled sheep anti-rabbit immunoglobulin G (IgG; Sigma-Aldrich, St Louis, MO, USA) diluted 1:200 with 10% NSS in PBS at 4°C with shaking and then washed numerous times at room temperature. Tissues were mounted in glycerol on microscope slides and observed under a Nikon epifluorescence microscope. PRXamide-like immunoreactivity (PRXa-LI) was mapped with the aid of a drawing tube attachment and images were obtained using confocal microscopy consisting of a helium-neon laser (543 nm line) and Zeiss LSM Image Browser software.

Tissues from fed and unfed insects, of identical age, were compared for intensity of staining, keeping settings on the confocal microscope constant. All insects, fed or unfed, were kept at room temperature until they were dissected. To ensure consistency in measurement of intensity of staining, images of individual immunoreactive cell bodies were taken keeping the nucleus of the cell in focus. Several post-feeding time points were compared to insects that had been exposed to the rabbit but not allowed to feed. Staining intensity was converted into grayscale values using the ImageJ Software (Rasband, 2005) and then subjected to statistical analyses including analysis of variance (ANOVA) and Tukey post-test. Grayscale values of confocal images were analyzed over an intensity scale from 0 to 255 (minimum to maximum intensity threshold for an 8-bit image, respectively).

Reverse phase high performance liquid chromatography (RP-HPLC)

Central nervous systems were dissected under saline and pooled in a 500 μl volume of methanol:acetic acid:water (90:9:1, by volume) and stored at –20°C for later use. The CNS tissue from 250 insects was then sonicated and centrifuged at 10 000 ***g*** for 10 min. The supernatant was collected and dried in a Speed Vac concentrator (Savant, Farmingdale, NY, USA) and then reconstituted in 0.1% trifluoroacetic acid (TFA). This sample was then applied to a C18 Sep-Pak cartridge (Waters Associates, Mississauga, ON, Canada) that had been sequentially equilibrated with 8 ml of methanol, 8 ml ddH_2O, 8 ml 0.1% TFA, and finally 5 ml 0.1% TFA containing 1 μg protease-free bovine serum albumin (BSA; Sigma, Mississauga, ON, Canada). Once the sample was loaded, the cartridge was first washed with 0.1% TFA and subsequently extracts were collected by eluting with 5 ml of 60% acetonitrile (ACN; Burdick and Jackson, Muskegon, MI, USA) with 0.1% TFA. The eluant was dried in a Speed Vac concentrator and then resuspended in high performance liquid chromatography (HPLC) start buffer (9% acetonitrile, 0.1% TFA) to be fractionated by reverse phase HPLC (RP-HPLC) using a Brownlee C18 column (Mandel/Alltech, Guelph, ON, Canada) with a linear gradient of 9–60% ACN over 34 min, beginning 5 min after injection. Fractions with Mas-CAPA-1-like

biological activity were identified by tubule secretion assays and cGMP RIA.

Malpighian tubule fluid secretion assay

Rhodnius prolixus have four Malpighian tubules (two bilateral pairs) composed of both upper and lower segments. Whole tubules from fifth instars were dissected under saline and transferred on glass probes to a Sylgard-coated Petri dish containing 20 μl drops of saline overlaid with water-saturated mineral oil. Two tubules were mounted in each 20 μl bathing droplet. The proximal end of the tubule was pulled out of the saline droplet and wrapped around a nearby minuten pin. The equilibrating saline was removed and replaced with saline containing 50 nmol l^{-1} 5-hydroxytryptamine (5-HT; Sigma, Oakville, ON, Canada) alone or combined with different concentrations of Mas-CAPA-1 (custom synthesized by GenScript Corp., Piscataway, NJ, USA) or CNS RP-HPLC fractions. Tubules were allowed to secrete for 30 min. Droplets of secreted fluid from the nicked end of the tubule were then collected using an oil-filled micropipette tip. The droplet was then blown out under oil to be measured on the bottom of the Sylgard-coated Petri dish. The droplet volume was calculated using the equation $V=(\pi/6)d^3$, where d is the droplet diameter measured using an eyepiece micrometer. At the end of the experiment, a maximal rate of secretion was established by stimulating with 1 μmol l^{-1} 5-HT to check on the viability of the tubules. Values, expressed as mean ± standard errors of the mean (s.e.m.), were then subjected to statistical analysis using Student's *t*-test.

Malpighian tubule cyclic GMP radioimmunoassay

Malpighian tubules were dissected under saline and tested as a set, including all four tubules from fifth instars, and transferred to a microcentrifuge tube containing saline, 50 nmol l^{-1} 5-HT alone or 50 nmol l^{-1} 5-HT combined with either Mas-CAPA-1 or CNS RP-HPLC fractions in a total volume of 50 μl. Tubules were incubated for 10 min and the experiment terminated by adding 250 μl of boiling 50 mmol l^{-1} sodium acetate (pH 6.2). The incubation tubes were then immediately placed in a boiling water bath for 5 min and then stored at –20°C. To prepare the samples for the assay, tubes were thawed, sonicated briefly on ice and centrifuged at 4°C for 10 min at 8800 ***g***. The supernatant was then collected and assayed using a cyclic GMP RIA kit (PerkinElmer/NEN, Boston, MA, USA). Assays were performed according to the manufacturer's instructions except for some minor changes in volumes and ratio of reagents.

Results

PRXamide-like immunoreactivity

Overview

In general, PRXamide-like immunoreactivity (PRXa-LI) in the central nervous system of *R. prolixus* is present in bilaterally paired cells and processes, as illustrated in the composite *camera lucida* drawings (Fig. 1). With the exception of strongly staining cells in the posterior-ventral mesothoracic ganglionic mass (MTGM), most processes could only be traced a short distance within the CNS. The processes, excluding those associated with the dorsal vessel and abdominal nerves, did not appear to exit the CNS. There were no differences observed in PRXa-LI between male and female fifth instar *R. prolixus*; however the intensity of immunoreactive staining differed greatly following a blood meal (see later). Overnight preincubation of the antiserum with Mas-CAPA-1 (50 μmol l^{-1}) eliminated all immunoreactivity of cells and processes within the CNS with the exception of the very intensely staining cells of the posterior-ventral MTGM, which were greatly reduced in intensity.

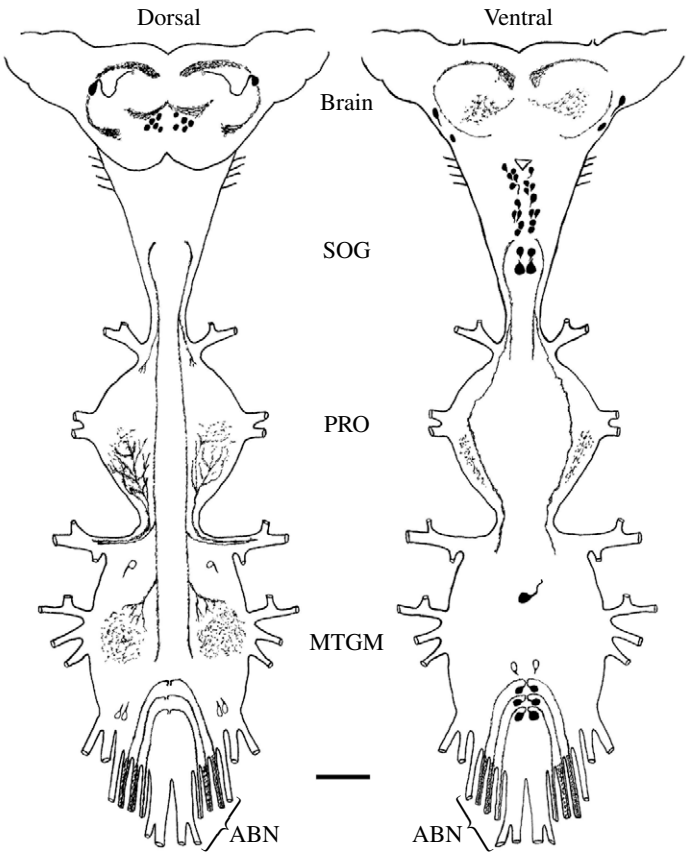

Fig. 1. Composite *camera lucida* drawing of PRXamide-like immunoreactive cells and processes in the central nervous system of *R. prolixus*. Left, dorsal view; right, ventral view. Filled cells indicate strong PRXamide-like immunoreactivity (PRXa-LI) and open cells indicate weak PRXa-LI. Within the mesothoracic ganglionic mass (MTGM), the ventral paired median cells give rise to immunoreactive processes which project dorsally and then exit the CNS *via* the second, third and fourth abdominal nerves (ABN) where they develop neurohaemal sites. PRO, prothoracic ganglion; SOG, sub-oesophageal ganglion. Scale bar, 200 μm.

Brain and retrocerebral complex

On the dorsal surface of the brain, two main groups of cells showed PRXa-LI (Fig. 2A). The first group consists of a

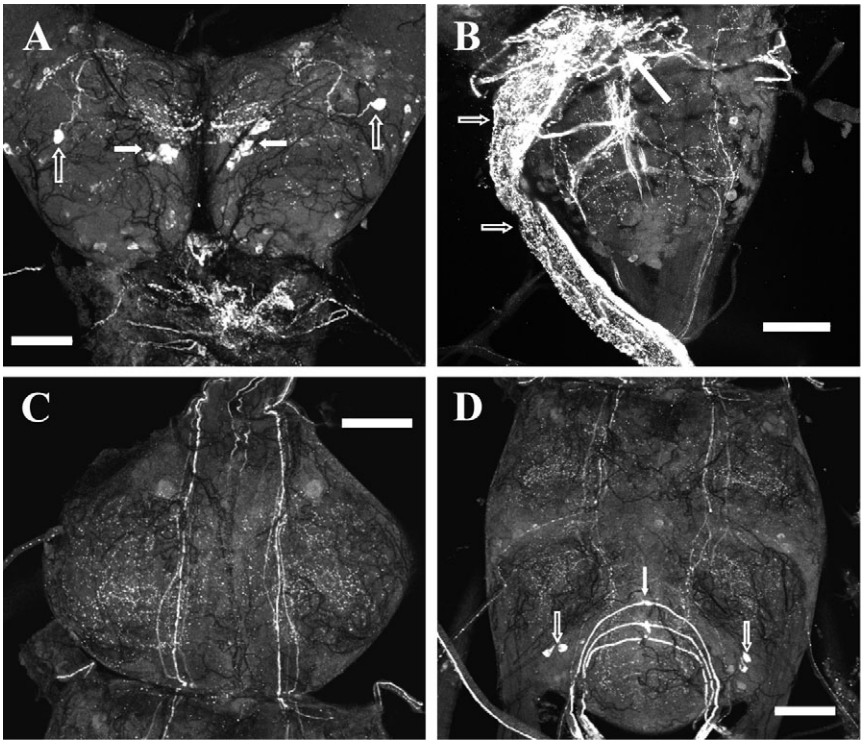

Fig. 2. PRXamide-like immunoreactivity (PRXa-LI) in fifth-instar *R. prolixus*. Dorsal views of (A) brain, (B) sub-oesophageal ganglion (SOG), (C) prothoracic ganglion and (D) the mesothoracic ganglionic mass (MTGM). In A, putative lateral neurosecretory cells and medial neurosecretory cells are indicated by open and filled arrows, respectively. In B, note the strong immunoreactivity in putative neurohaemal sites on the dorsal vessel (open arrows) and light staining over the corpus cardiacum (filled arrow). In C, note the numerous medial processes with PRXa-LI that originate in the SOG and project into the MTGM. In D, note the lateral paired cells (open arrows) and the processes originating from the ventral paired neurosecretory cells (filled arrows). Scale bar, 100 μm.

bilateral pair of lateral neurosecretory cells (LNCs) prominently identified in the border region of the optic lobe and brain, which have processes projecting medially through the brain. A second group of cells consists of five pairs of medial neurosecretory cells (MNCs) arranged as a cluster along the boundary between the protocerebral lobes. Immunoreactive varicosities were also present along the periphery of the protocerebral lobes originating at the optic lobe/brain boundary and also present anterior to the MNCs. Some immunoreactivity appeared to be associated with the corpus cardiacum, however, extensive PRXamide-like immunoreactive processes were present along the walls of the aorta, decreasing in intensity as they proceed posteriorly (Fig. 2B).

The ventral surface of the brain contains two sets of bilaterally paired cells (Fig. 3A), which are located posterior to the LNCs. These cells project processes posteriorly. Varicosities similar to the pattern visible on the dorsal surface of the brain were also observed on the ventral surface of the brain. Immunoreactive processes were also seen in the recurrent nerve with numerous cell bodies staining for PRXa-LI in the frontal ganglion (Fig. 4A).

Sub-oesophageal ganglion (SOG)

The dorsal sub-oesophageal ganglion (SOG) did not contain any PRXamide-like immunoreactive cell bodies; however, two bilaterally paired immunoreactive processes were observed passing in the medial and lateral margins of the SOG (Fig. 2B). On the ventral SOG, several bilaterally paired cells demonstrate strong PRXa-LI. Some of these cells can be seen projecting processes posteriorly in Fig. 3B. PRXamide-like immunoreactive processes were present at the posterior margin of the SOG and within the connectives of the SOG and prothoracic ganglion.

Prothoracic ganglion (PRO)

Processes originating from the SOG are observed in the dorsal prothoracic ganglion (PRO). The medial processes continue through to the posterior of the PRO whereas some lateral processes arborise in the central neuropile (Fig. 2C). On the ventral surface of the PRO, some faint PRX-amide immunoreactive staining was observed in the central neuropile (Fig. 3C).

Mesothoracic ganglionic mass (MTGM)

On the dorsal surface of the mesothoracic ganglionic mass (MTGM), a small number of cell bodies showed faint PRXa-LI in both the mesothoracic and the abdominal neuromeres (Fig. 2D). Specifically, in the mesothoracic neuromere there were two cells (bilaterally paired) with posteriorly projecting processes. In the abdominal neuromeres, there are four cells (two bilaterally paired) along the lateral margins with processes projecting medially. The processes originating from the SOG and passing through the PRO continue into the MTGM where they arborise in the metathoracic neuromere. Processes from three pairs of strongly staining cell bodies located on the ventral MTGM project dorsally and then posteriorly continue into the second, third and fourth abdominal nerves that stem from the abdominal neuromeres in the posterior MTGM (Fig. 2D, Fig. 3D). These immunoreactive processes form extensive neurohaemal sites over the proximal portion of the nerves and distally continue as fine processes (Fig 3D, Fig. 4B).

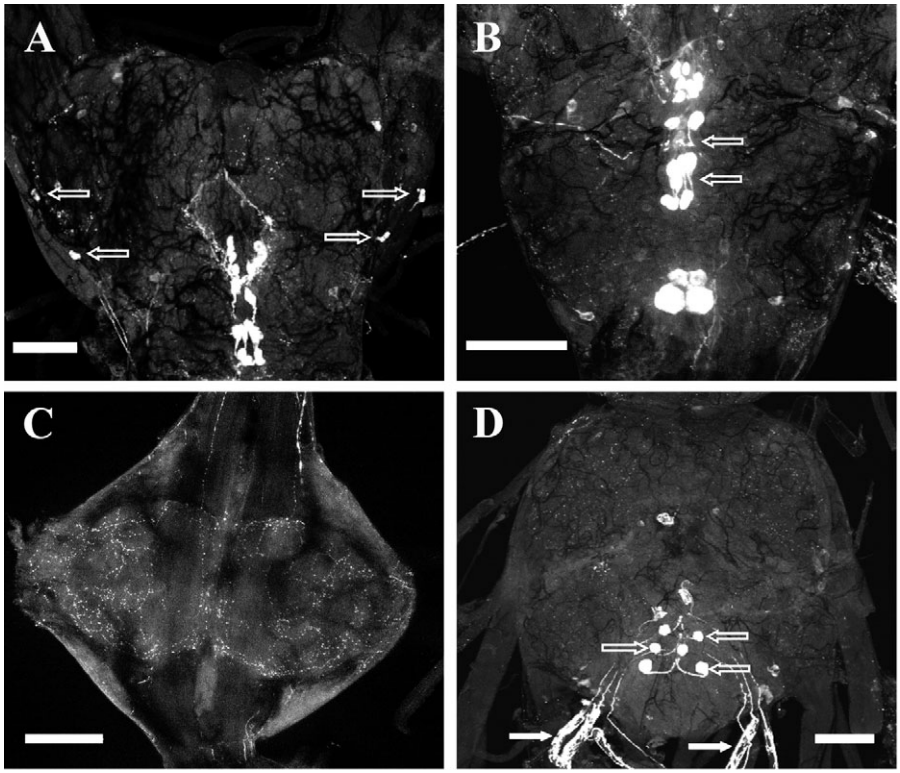

Fig. 3. PRXamide-like immunoreactivity (PRXa-LI) in fifth-instar *R. prolixus*. Ventral views of (A) brain, (B) sub-oesophageal ganglion, (C) prothoracic ganglion (PRO) and (D) the mesothoracic ganglionic mass. In A, note the lateral immunoreactive cells (open arrows). In B, note the numerous bilaterally paired cell bodies (open arrows) lying medially. In C, note the lightly staining immunoreactive varicosities over the ventral PRO. In D, note the immunoreactive cell bodies (open arrows) and extensive neurohaemal-like immunoreactivity on the abdominal nerves (closed arrows). The intensity of staining of these cells is greatly reduced 3–4 h following feeding (see later). Scale bar, 100 μm.

Cell bodies with variable PRXa-LI were observed on the ventral surface of the MTGM. Beginning anteriorly, there was a ventral unpaired medial (VUM) neuron within the mesothoracic neuromere, which had strong PRXa-LI. Moving posteriorly, within the metathoracic neuromere, a bilateral pair of cells showed weak PRXa-LI. Finally, within the midline of the abdominal neuromeres, the six (three bilaterally paired) strongly staining cells, referred to earlier, were consistently seen with their processes projecting dorsally and then posteriorly out of corresponding abdominal nerves. Of this strongly staining group, the most anterior pair projects through the second abdominal nerve, whereas the second pair project to the third abdominal nerves, and the last strongly staining pair, and most posterior, project processes through the fourth abdominal nerves. Each cell of the most posterior pair have a diameter of 29 μm, which is considerably larger than the two more anterior pairs of cells (16 μm).

Time-course immunohistochemical analysis

Following a blood meal, extensive changes in the intensity of staining of PRXamide-like cells and processes are observed. Specifically, over the MTGM, the staining of the six strongly staining cells within the abdominal neuromeres become weaker in intensity, beginning as early as 3 h following a blood meal (Fig. 5). These cells project into the abdominal nerves, and form neurohaemal sites, suggesting a location for release into the haemolymph. Changes in PRXa-LI post-feeding were analyzed by evaluating staining intensity of the six strongly staining cell bodies over the MTGM using the ImageJ software package. No significant changes in staining intensity were seen

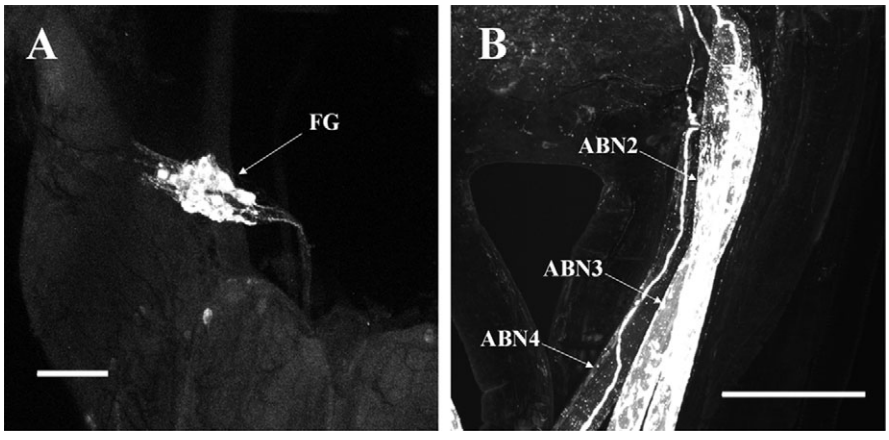

Fig. 4. PRXamide-like immunoreactivity (PRXa-LI) in fifth-instar *R. prolixus*. (A) Frontal ganglion (FG) with numerous immunoreactive cell bodies; (B) immunoreactive neurohaemal sites on the second (ABN2), third (ABN3) and fourth (ABN4) abdominal nerves. In B, note that abdominal nerves two and three contain more elaborate immunoreactive neurohaemal sites. Scale bar, 100 μm.

in unfed control animals across all time points analyzed. In contrast, staining intensity appears to weaken as early as 3 h following feeding (see Fig. 5). As time post-feeding progresses, significant decreases in staining intensity were observed. Interestingly, the largest and most posterior pair of cells regained staining before the two smaller and more anterior pairs of cells. This last pair of cells project processes into the fourth abdominal nerves, which has notably fewer neurohaemal sites than the second and third abdominal nerves. Nonetheless, the intensity of staining of the neurohaemal sites and processes over the abdominal nerves was visibly assessed and was reduced at the same time points.

Malpighian tubule fluid secretion assay

To better characterize the anti-diuretic mechanism in *R. prolixus*, we tested various concentrations of Mas-CAPA-1 on

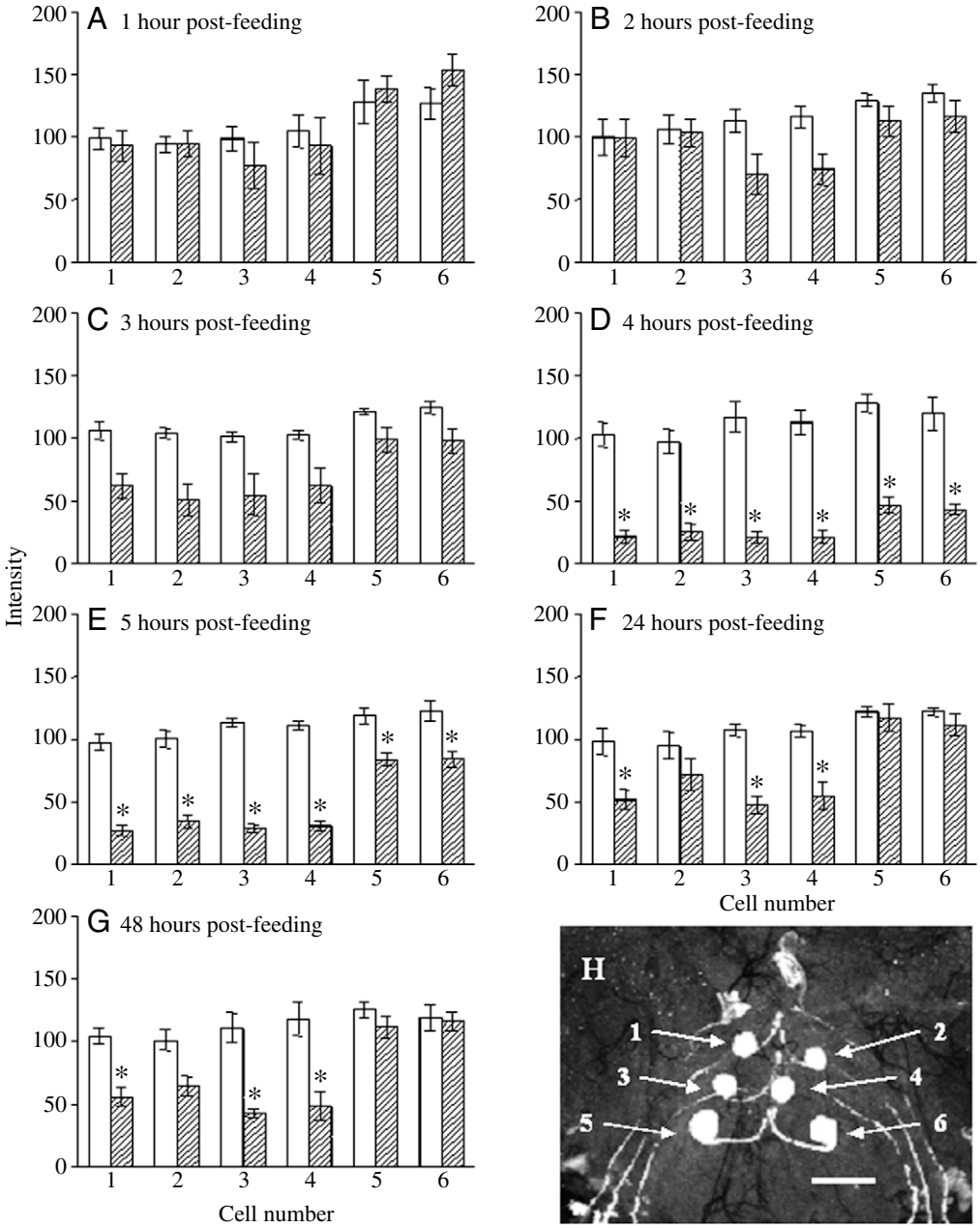

Fig. 5. Time-course immunohistochemical analysis of the ventral paired medial neurosecretory cells in the MTGM of fifth-instar *R. prolixus*. Immunohistochemical analysis was conducted on a group of animals that were either fed for 20 min on rabbit's blood (hatched bars) or not fed (white bars). (A–G) PRXamide-like immunoreactivity (PRXa-LI) was examined at 1, 2, 3, 4, 5, 24 and 48 h post feeding, respectively. (H) Confocal image of the ventral paired median neurosecretory cells showing the labelling scheme utilized in A–G. Scale bar, 50 μm. *PRXa-LI that differs significantly from controls (unfed) (P<0.05, ANOVA and Tukey post-test).

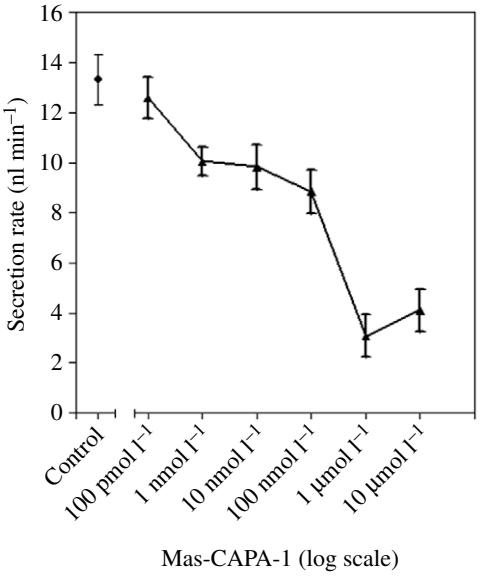

Fig. 6. Dose–response curve demonstrating Mas-CAPA-1 inhibition of secretion by Malpighian tubules stimulated with 50 nmol l^{-1} 5-HT. Control tubules received 50 nmol l^{-1} 5-HT. Values are mean ± s.e.m., *N*=8 or more tubules.

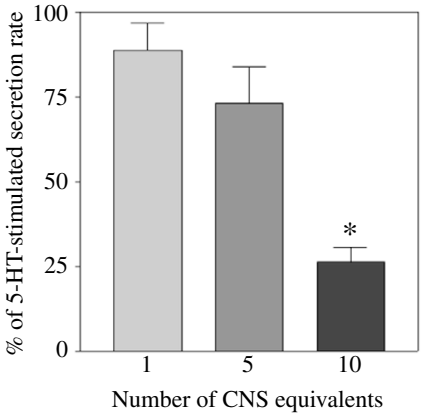

Fig. 7. Inhibition of secretion (stimulated with 50 nmol l^{-1} 5-HT) with increasing doses of Fraction 25 from RP-HPLC. Values are expressed as a percentage of control (normal secretion of tubules stimulated with 50 nmol l^{-1} 5-HT). Values are mean ± s.e.m., *N*=8 or more tubules. *Statistically significant inhibition (P<0.001).

5-HT-stimulated tubules and observed a dose-dependent inhibition on tubule secretion (Fig. 6). Threshold was observed at approximately 0.1 nmol l^{-1} Mas-CAPA-1 and maximal inhibition at a dose of 1 μmol l^{-1} Mas-CAPA-1. In order to provide further empirical evidence for the presence of a Mas-CAPA-1-like neuropeptide in *R. prolixus*, we tested individual fractions from RP-HPLC against 5-HT-stimulated tubules and identified an anti-diuretic fraction. This fraction (fraction 25) ran in close proximity to synthetic Mas-CAPA-1, which eluted from the C18 column at 24.5 min (an acetonitrile concentration of 38.25%). Tubules incubated in this fraction, in the presence of 50 nmol l^{-1} 5-HT, showed a dose-dependent decrease in secretion rate (Fig. 7). Thus, tubules stimulated with 50 nmol l^{-1} 5-HT and this anti-diuretic fraction from 1 CNS equivalent inhibited secretion by 11%, from 5 CNS equivalents by 27% and from 10 CNS equivalents by 74%. The ability of a single CNS equivalent to decrease secretion by only 11% could be due to the combined influence of losses associated with sonication of tissues and preparatory steps prior to HPLC as well as impurity of the factor. Since CNS extracts were run only through a single column, other factors eluting within this fraction could be contributing to the biological activity observed.

Malpighian tubule cyclic GMP radioimmunoassay

To further understand the mechanism of action of this endogenous Mas-CAPA-1-like anti-diuretic neuropeptide in *R. prolixus*, cyclic GMP radioimmunoassays were conducted on fifth instars to confirm the previous observation of an elevation of intracellular cGMP in response to Mas-CAPA-1 in third-instar tubules stimulated with 5-HT. 5-HT (50 nmol l^{-1}) lowered cGMP levels of fifth-instar tubules and these levels were restored to control values by Mas-CAPA-1 at 500 nmol l^{-1} (Fig. 8). Similarly, fraction 25 at 10 CNS equivalents also increased the cGMP levels of 5-HT-stimulated tubules (Fig. 8). The levels of cGMP in tubules stimulated with fraction 25, in the presence of 50 nmol l^{-1} 5-HT, were found to be significantly higher than unstimulated tubules, suggesting that the actions of this endogenous Mas-CAPA-1-like factor involve augmenting levels of intracellular cGMP.

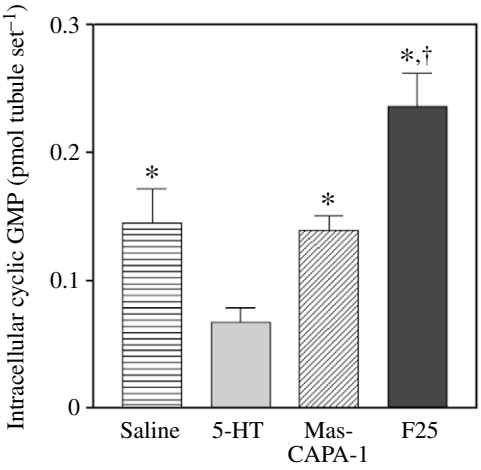

Fig. 8. Change in levels of intracellular cGMP in tubules stimulated with 5-HT alone or in combination with Mas-CAPA-1 (500 nmol l^{-1}) or Fraction 25 (F25; 10 CNS equivalents) *vs* saline alone. 5-HT lowers cGMP levels, and these levels can be restored to those with saline alone or above by Mas-CAPA-1 or Fraction 25 (*significantly different from 5-HT alone at P<0.05). In addition, levels of intracellular cGMP in tubules stimulated with Fraction 25 were also significantly higher (†) than tubules stimulated with Mas-CAPA-1 or saline alone (P<0.05).

Discussion

Using a polyclonal antiserum to MasPETH, which identifies peptides sharing a common C-terminal tripeptide motif PRXamide [X=I, L, M or V (Žitnaň et al., 2003)], we have demonstrated the presence of cell bodies and processes having PRXa-LI throughout the central and peripheral nervous system of fifth-instar *R. prolixus*. Several insect neuropeptides sharing this common C-terminal motif have been identified and the CAPA gene codes three extended PRXamides, including CAPA-1 (a PRVamide). Of these closely related neuropeptides, the PRXa-LI observed in fifth-instar *R. prolixus* closely resembles the immunocytochemical localization of the CAPA gene peptides in *D. melanogaster* (Kean et al., 2002). More specifically, the strongly immunoreactive ventral medial cells over the abdominal neuromeres in the posterior MTGM closely resemble three pairs of abdominal neurosecretory cells in *D. melanogaster*, which stain for the CAPA precursor protein, and thus provides evidence that these cell types produce CAP2b-related peptides (Kean et al., 2002). In addition, the distribution in *R. prolixus* of PRXamide-like immunoreactive cells over the MTGM resembles CAPA gene-expressing cells in the abdominal ganglia of larval and adult *M. sexta* (Loi and Tublitz, 2004). Medial neurosecretory cells showing PRXa-LI over the dorsal brain of fifth-instar *R. prolixus* resemble cells over the dorsal brain of *M. sexta* larvae that express CAPA transcripts (Loi and Tublitz, 2004). Taken together, these similarities in immunoreactivity along with the abolition of immunoreactivity following preincubation of the antiserum with Mas-CAPA-1, suggests that the immunoreactivity observed indicates the presence of Mas-CAPA-1-like neuropeptides in the CNS of *R. prolixus*. More importantly, the medial ventral cell bodies in the MTGM project processes into the abdominal nerves, well-known neurohaemal release sites (Miksys and Orchard, 1994), and thus provides a location for release of these peptides into the haemolymph. Furthermore, the intensity of staining of immunoreactivity in these cell bodies and their neurohaemal release sites is greatly reduced 3–4 h post feeding – a time when the cessation of diuresis is observed (Maddrell, 1964), suggesting the release of an anti-diuretic factor.

We suggest that the strong PRXa-LI observed over the MTGM and abdominal nerves provides evidence for a CAPA-like neuropeptide in the CNS of *R. prolixus*, which includes a Mas-CAPA-1-like peptide. The presence of PRXamide-like immunoreactive cell bodies in addition to processes and neuropiles over the length of the CNS suggest additional roles as neurotransmitters and/or neuromodulators. Future studies will help elucidate whether the PRXamide-like peptide functions as a neurotransmitter and/or neuromodulator in *R. prolixus*. Certainly, the results indicate that the PRXamide-like neuropeptide in *R. prolixus* acts as a neurohormone since there is evidence of release from the MTGM and abdominal nerves as well as activity on a non-innervated visceral tissue (Malpighian tubules).

Previous analyses on third-instar *R. prolixus* tubules showed dose-dependent effects of Mas-CAPA-1in the nanomolar range (Quinlan et al., 1997). To better understand the response of tubules to Mas-CAPA-1-like peptides in fifth-instar *R. prolixus,* we tested a broad range of physiological doses of Mas-CAPA-1 to determine the dose-dependency on isolated tubules. At 0.1 nmol l^{-1}, the lowest dose tested, Mas-CAPA-1 caused a 5% decrease in secretion. This neuropeptide had a maximal effect on tubules at 1 μmol l^{-1}, inhibiting secretion by over 75%. A higher dose (10 μmol l^{-1}) of this neuropeptide was slightly less effective at inhibiting fluid secretion, possibly indicating the beginning of receptor desensitization.

Further evidence for the presence of a Mas-CAPA-1-like neuropeptide in *R. prolixus* sharing similar characteristics to Mas-CAPA-1 was revealed by bioassay of native material. Analysis of RP-HPLC fractions from 250 CNSs revealed a factor with anti-diuretic effects on Malpighian tubules stimulated with 5-HT. This factor eluted from the C18 column at a similar time and acetonitrile concentration to Mas-CAPA-1, suggesting that this factor shares similar chromatographic properties to Mas-CAPA-1. Doses as low as a single CNS equivalent were adequate in eliciting an anti-diuretic effect on tubules. Furthermore, tubules stimulated with higher doses of this factor demonstrated a greater inhibition of secretion, indicating the effects of this factor are dose dependent. To our knowledge, this is the first study to show direct evidence for the presence of an endogenous anti-diuretic factor in *R. prolixus*, which significantly inhibits 5-HT-stimulated secretion in a dose-dependent manner.

This same fraction elevated intracellular cyclic GMP levels in tubules stimulated with 5-HT, indicating that this second messenger may be exploited by the native Mas-CAPA-1-like anti-diuretic peptide in *R. prolixus*. Moreover, this fraction not only reversed the effects of 5-HT on cGMP, but at this dose also increased cGMP above its original saline control values. This result implies that this factor is actively involved in the synthesis of intracellular cGMP, which, as suggested previously, may involve the actions of a guanylate cyclase belonging to the class of membrane-bound enzymes (Quinlan et al., 1997). Interestingly, Mas-CAPA-1 has been shown to increase the synthesis of nitric oxide and cGMP leading to an increase in fluid production in *D. melanogaster* tubules (Davies et al., 1995; Davies et al., 1997). Expression of the receptor for Mas-CAPA-1 in tubules has been shown in a number of dipterans (see Pollock et al., 2004). It is interesting that there has been a divergence in signalling between these organisms.

In conclusion, this study investigated the distribution of PRXa-LI throughout the CNS of *R. prolixus*. It is probable that many of these cells, especially those in the abdominal neuromeres, are Mas-CAPA-1-like since: (1) preincubation of the antiserum with Mas-CAPA-1 peptide eliminated all immunoreactivity within the CNS; (2) immunoreactivity was significantly reduced beginning 3–4 h post-feeding in accordance with the time of anti-diuretic behaviour (Maddrell, 1964), which suggests the release of an anti-diuretic peptide from the putative neurohaemal release sites on the abdominal nerves; (3) this study, as well as previous studies on third-instar *R. prolixus*, have shown that Mas-CAPA-1 elicits an anti-

diuretic effect on *R. prolixus* tubules (Quinlan et al., 1997); (4) tubule secretion assay utilizing CNS fractions from a C18 HPLC run identified a factor with Mas-CAPA-1-like biological activity, which inhibits 5-HT-induced tubule secretion; lastly, (5) this same RP-HPLC fraction containing an anti-diuretic factor was also effective at increasing levels of intracellular cGMP in Malpighian tubules.

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