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A NEURONAL ROLE FOR A CRUSTACEAN RED PIGMENT CONCENTRATING HORMONE-LIKE PEPTIDE: NEUROMODULATION OF THE PYLORIC RHYTHM IN THE CRAB, *CANCER BOREALIS*

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SUMMARY

The distribution of red pigment concentrating hormone (RPCH)-like immunoreactivity (RPLI) in the stomatogastric nervous system of the crab, *Cancer borealis*, was studied using whole-mount immunocytochemistry. RPLI was seen in neuropilar processes in the stomatogastric ganglion (STG), and in somata in the oesophageal ganglion and commissural ganglia. Staining was blocked by preincubating the antiserum with RPCH, as well as with a number of adipokinetic hormones (AKHs) and related peptides.

Synthetic RPCH had strong actions on the pyloric rhythm of the isolated STG. Bath applications of RPCH (10^{-9}–10^{-6} mol l^{-1}) increased the cycle frequency in preparations displaying slow pyloric rhythms, and initiated rhythmic pyloric activity in silent preparations. In the presence of tetrodotoxin (TTX), RPCH evoked rhythmic non-impulse-mediated alternations in membrane potential in the lateral pyloric and pyloric dilator motor neurones. The effects of RPCH were compared to those of a series of AKHs which resemble RPCH structurally.

The immunocytochemical and physiological data together suggest that RPCH or a similar molecule is a neurally released modulator of the STG.

INTRODUCTION

Many neuropeptides were first identified because of their presence in peripheral tissues and organs and their action upon them, and subsequently were found in the nervous system where they are thought to function as transmitters and modulators (Krieger, Brownstein & Martin, 1983). The hormones that control pigment translocation in crustaceans have been extensively studied for most of this century (Perkins & Snook, 1931; Josefsson, 1983; Rao, 1985). In particular, red pigment concentrating hormone (RPCH) was identified originally by its ability to concentrate red pigment granules within the erythrophores of many different crustaceans,

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including the crabs *Uca pugilator*, *Cardisoma carnifex* and *Ocypode pallidula* (Cooke, Haylett & Weatherby, 1977; Josefsson, 1983; Rao, 1985). More recently, RPCH has been implicated in the concentration of pigments in the crustacean ommatidia (Kulkarni & Fingerman, 1986).

The amino acid sequence of RPCH (pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH_2) was determined originally from the shrimp *Pandulus borealis* (Fernlund & Josefsson, 1972). An apparently identical RPCH has been identified in the shrimp *Leander adspersus* (Carlsen, Christensen & Josefsson, 1976) and in the crab *Cardisoma carnifex* (Newcomb, 1983). The RPCH-like peptide from the crab *Carcinus maenas* appears similar but not identical to the shrimp peptide (Mangerich, Keller & Dircksen, 1986). Interestingly, the sequence of RPCH is quite similar to those of the adipokinetic hormones (AKHs) in insects, although RPCH and the AKHs are thought to serve different physiological functions. In addition to RPCH, the RPCH/AKH peptide family includes at least seven structurally distinct peptides (Jaffe *et al.* 1986; Schaffer, 1986; Gade & Rinehart, 1987). Two of these (AKH IIS and AKH IIL) differ from RPCH by only a single amino acid. Like RPCH and the AKH IIs, M I (CC1) and M II (CC2) are also octapeptides. The other three identified members of this peptide family are slightly longer: M-AKH is a nonapeptide while AKH I and HTF II are decapeptides. The adipokinetic hormones are known to increase lipid mobilization in insect muscle during flight (Stone, Mordue, Batley & Morris, 1976), enhance hyperglycaemic activity and effect cardioacceleration in insects (Baumann & Gersch, 1982; Scarborough *et al.* 1984) and increase myoactivity in insect skeletal muscle (O'Shea, Witten & Schaffer, 1984). An RPCH/AKH-like peptide with cardioexcitatory activity has also been isolated from the clam *Mercenaria mercenaria* (Greenberg *et al.* 1985).

In crustaceans, a major source of hormonally released RPCH is the sinus gland, a neurohaemal organ in the crustacean eyestalk (Cooke *et al.* 1977; Cooke & Haylett, 1984; Rao, 1985). Several regions of the crustacean central nervous system also contain RPCH, or an RPCH-like peptide, including the brain, thoracic ganglia and the commissural ganglia (Rao, 1985; Mangerich *et al.* 1986). Recently, the distributions of RPCH-like immunoreactivity (RPLI) and AKH-like immunoreactivity have been studied in several crustacean species (Madsen, Herman & Elde, 1985; Mangerich *et al.* 1986; Schooneveld, van Herp & van Minnen, 1987). These studies show that the central nervous systems of crustaceans contain a peptide identical, or similar, to RPCH as well as an AKH-like peptide (Madsen *et al.* 1985; Mangerich *et al.* 1986; Schooneveld *et al.* 1987), and prompted the suggestion that an RPCH-like peptide also functions as a neurotransmitter or neuromodulator (Mangerich *et al.* 1986; Schooneveld *et al.* 1987). We now provide physiological evidence that this is the case.

The crustacean stomatogastric ganglion (STG) contains only 30 neurones, and produces two different motor patterns that control the movements of the animal's stomach (Selverston & Moulins, 1987). Previous work has shown that a large number of amines and peptides are present in inputs to the STG (Marder, 1984, 1987) and that these substances can influence the motor pattens produced by the STG

(Marder, 1984, 1987; Marder, Hooper & Eisen, 1987*b*; Harris-Warrick & Flamm, 1986). Using an antiserum against RPCH (Madsen *et al.* 1985) we find RPLI in input fibres to the STG and in fine processes within the STG neuropile. Moreover, we find that exogenously applied RPCH has strong excitatory effects on the pyloric rhythm produced by the STG. Thus RPCH or a similar peptide is likely to be a neurotransmitter or modulator in the stomatogastric nervous system. It appears, therefore, that RPCH can play both a systemic hormonal role in the control of pigment translocation and a neurotransmitter/modulator role in the nervous system. Some of these data have been presented in abstract form (Nusbaum & Marder, 1986).

MATERIALS AND METHODS

Animals

Cancer borealis were obtained from local (Boston) commercial fishermen, and held in artificial seawater aquaria until used. 95 male animals weighing between 100 and 500 g were used (68 for immunocytochemistry; 27 for electrophysiology).

Solutions

C. borealis physiological saline was as reported by Marder, Hooper & Siwicki (1986). All peptide-containing solutions were made directly before use at the desired concentrations from stock solutions that were kept frozen in small samples. Peptides were obtained from Peninsula or Bachem. Several of the stock solutions were prepared by first dissolving the peptide in 5–8% dimethylsulphoxide (DMSO). Control experiments showed that the trace amounts of DMSO applied to the physiological preparations had no effect on activity.

Anatomy and immunocytochemistry

Whole-mount immunocytochemistry on the stomatogastric nervous system was performed by the method of Beltz & Kravitz (1983) as described previously (Marder *et al.* 1986; Marder, Calabrese, Nusbaum & Trimmer, 1987*a*). The primary antiserum, a rabbit anti-RPCH antibody raised against RPCH coupled to thyroglobulin, was a gift from the Elde laboratory (Madsen *et al.* 1985). Incubations in the primary antiserum were made at 1:200 in triton–azide-containing buffer (Marder *et al.* 1986), containing 10% non-immune goat serum, for 24 h at 4°C. The secondary antiserum was Cappell rhodamine-labelled goat anti-rabbit used at 1:25. Preincubation blocks were performed by incubating the primary antibody with the peptide for 2 h at room temperature before applying the mixture to the preparation. Lucifer Yellow backfills were carried out as described by Marder *et al.* (1987*a*). Preparations were viewed using a Zeiss IM35 fluorescence microscope equipped with rhodamine and fluorescein filters. Colchicine-treated animals were injected with 15 mg ml^{-1} colchicine in physiological saline. Animals were sacrificed 2–4 days later, and tissues were processed as usual.

Physiology

Physiological experiments used routine methods for the STG. Extracellular recordings were made with Vaseline-insulated stainless steel pin electrodes. Intracellular recordings were made with 20–50 MΩ microelectrodes filled with potassium acetate. All experiments were carried out using a continuously flowing superfusion system (6–10 ml min^{-1}) cooled to 12–14°C that allowed rapid changing of the bath solution. Peptides were introduced into the bath without interrupting the flow of the solution. Bath volume was 15–20 ml.

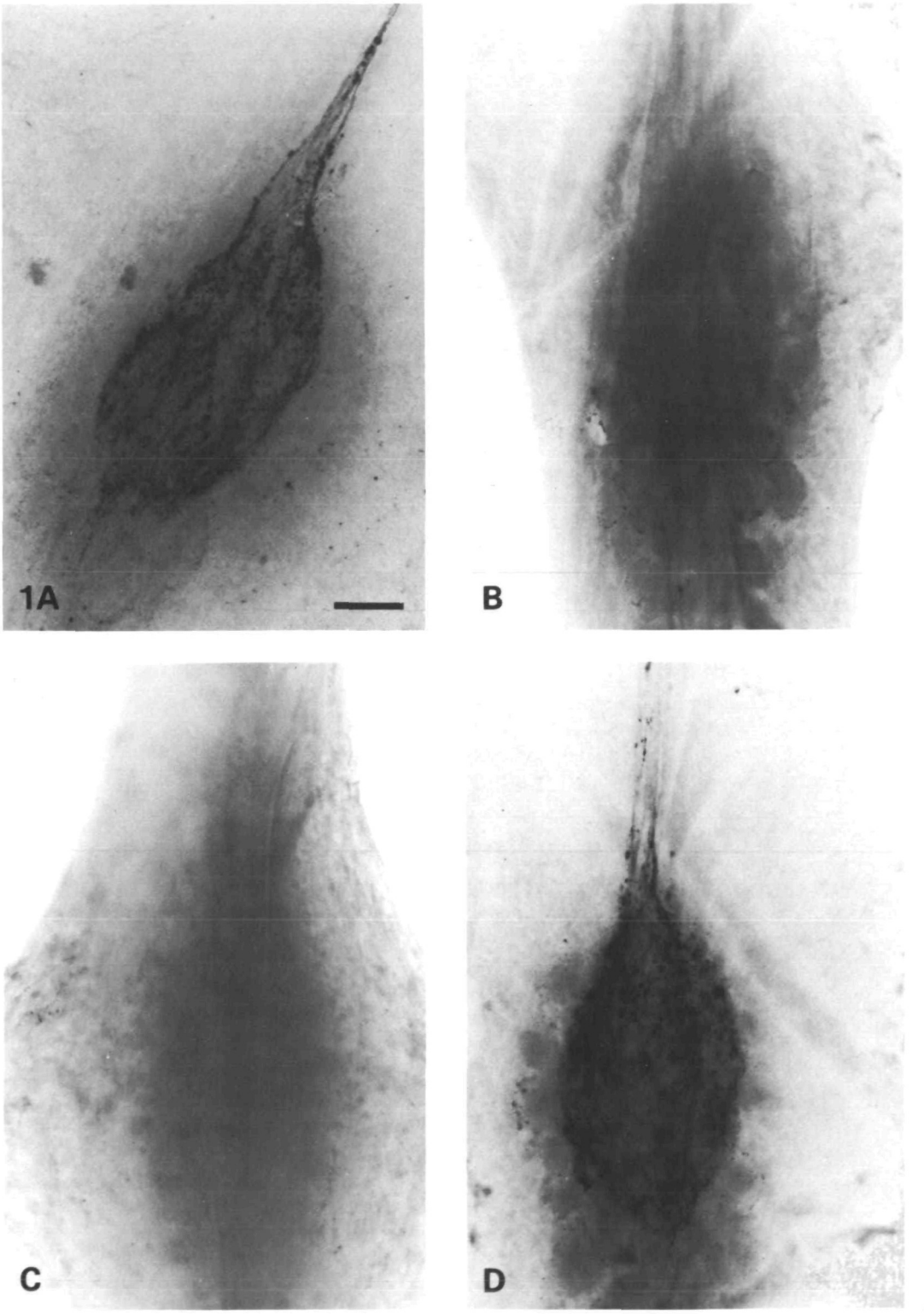
RESULTS

Distribution of RPLI in the stomatogastric nervous system

The 30 somata of the crab STG are about 50–100 μm in diameter and encircle a flat, central neuropile in which synaptic contacts are made. When the STG was processed by whole-mount immunocytochemistry (Beltz & Kravitz, 1983) using antibody raised against RPCH (Madsen *et al.* 1985), RPLI was observed exclusively in fine fibres and swellings throughout the neuropile (Fig. 1A). No somata in the STG were stained, nor was any RPLI seen in the motor nerves of the STG. Several fibres were seen in the stomatogastric nerve (STN), the nerve that brings inputs to the STG from the rest of the nervous system.

The specificity of the RPLI stain was studied using the ability of the antibody to label STGs subsequent to preincubation with different peptides. Preincubation of the antiserum with 10^{-6} mol l^{-1} RPCH abolished staining (Fig. 1B), as did preincubation with 10^{-6} mol l^{-1} AKH IIL (Fig. 1C) and 10^{-6} mol l^{-1} AKH IIS (not shown). In contrast, a much higher concentration of AKH I (10^{-4} mol l^{-1}) (Fig. 1D) was relatively ineffective in blocking the staining seen with antiserum to RPCH. The AKH peptides M I and M II blocked staining at 10^{-5} mol l^{-1}. RPLI staining was not blocked by preincubation with thyroglobulin (1 mg ml^{-1}), to which RPCH was coupled in generating the primary antibody. RPLI was also not blocked by preincubation with proctolin, FMRFamide or substance P (10^{-4} mol l^{-1}). These peptides were chosen because previous work has shown that these peptides or immunologically related ones are also present in the neuropile of the STG (Marder *et al.* 1986, 1987*a*; Goldberg, Nusbaum & Marder, 1986).

Fig. 1. RPLI in the stomatogastric ganglion (STG). (A) Whole-mount of the STG showing RPLI. Fine neuropilar processes in the STG and several input fibres in the stomatogastric nerve (upper right) are visible. No somata within the STG were labelled, but these somata can be faintly seen. (B–D) Preabsorption controls of RPLI in the STG using 10^{-6} mol l^{-1} RPCH (B) and 10^{-6} mol l^{-1} AKH IIL (C) completely block staining while RPLI persists despite 10^{-4} mol l^{-1} AKH I (D). The small punctate fluorescence in B and C, especially prominent around the perimeter of the STG somata, does not represent RPLI, but is characteristic of an autofluorescence sometimes also present in STGs that have not been incubated with any primary antiserum. Calibration bar for A–D, 100 μm. Photographs were printed on Kodak Panalure paper from Ektachrome 400 colour slides. Therefore, signal is viewed as black.


1A
B
C
D

Some of the fibres that project into the STG come from neurones with their somata either in the single oesophageal ganglion (OG) or the paired commissural ganglia (CGs) (Selverston & Moulins, 1987). Therefore, we determined the distribution of RPLI in the OG and CGs, and then attempted to determine the location of the source of the RPLI projections into the STG neuropile. For these latter experiments we combined Lucifer Yellow backfills of individual nerves with subsequent immunolabelling of the same tissue using the antiserum against RPCH, visualized with a rhodamine-conjugated secondary antibody (see Marder *et al.* 1987*a*).

Two of the approximately 14 OG neurones showed RPLI. The Lucifer Yellow backfill–RPLI double-label technique showed that the two RPLI neurones in the OG send axons towards the brain *via* the inferior ventricular nerve (IVN), rather than into the STN towards the STG (Fig. 2A,B). Interestingly, these same two neurones also showed FMRFamide-like immunoreactivity (Marder *et al.* 1987*a*; Callaway, Masinovsky & Graubard, 1987) and SCP_b-like immunoreactivity (Callaway *et al.* 1987).

The CGs contain about 400 neurones each. The CGs showed extensive neuropilar staining, and 5–10 somata showed RPLI (Fig. 2C). These somata were visualized most effectively after colchicine treatment, which precluded combining Lucifer Yellow backfills with immunocytochemistry for these cells. Thus, although these neurones are good candidates for the source of the RPLI in the STG neuropile, we have been unable to demonstrate this directly. The commissures connect the brain to the suboesophageal ganglion. RPLI was found in individual fibres within the commissures (Fig. 2D). Some of these fibres were seen to ramify within the CGs, which are outpocketings of the commissures. The distribution of RPLI throughout the complete stomatogastric nervous system and the position of the more anterior ganglia are shown in Fig. 2E.

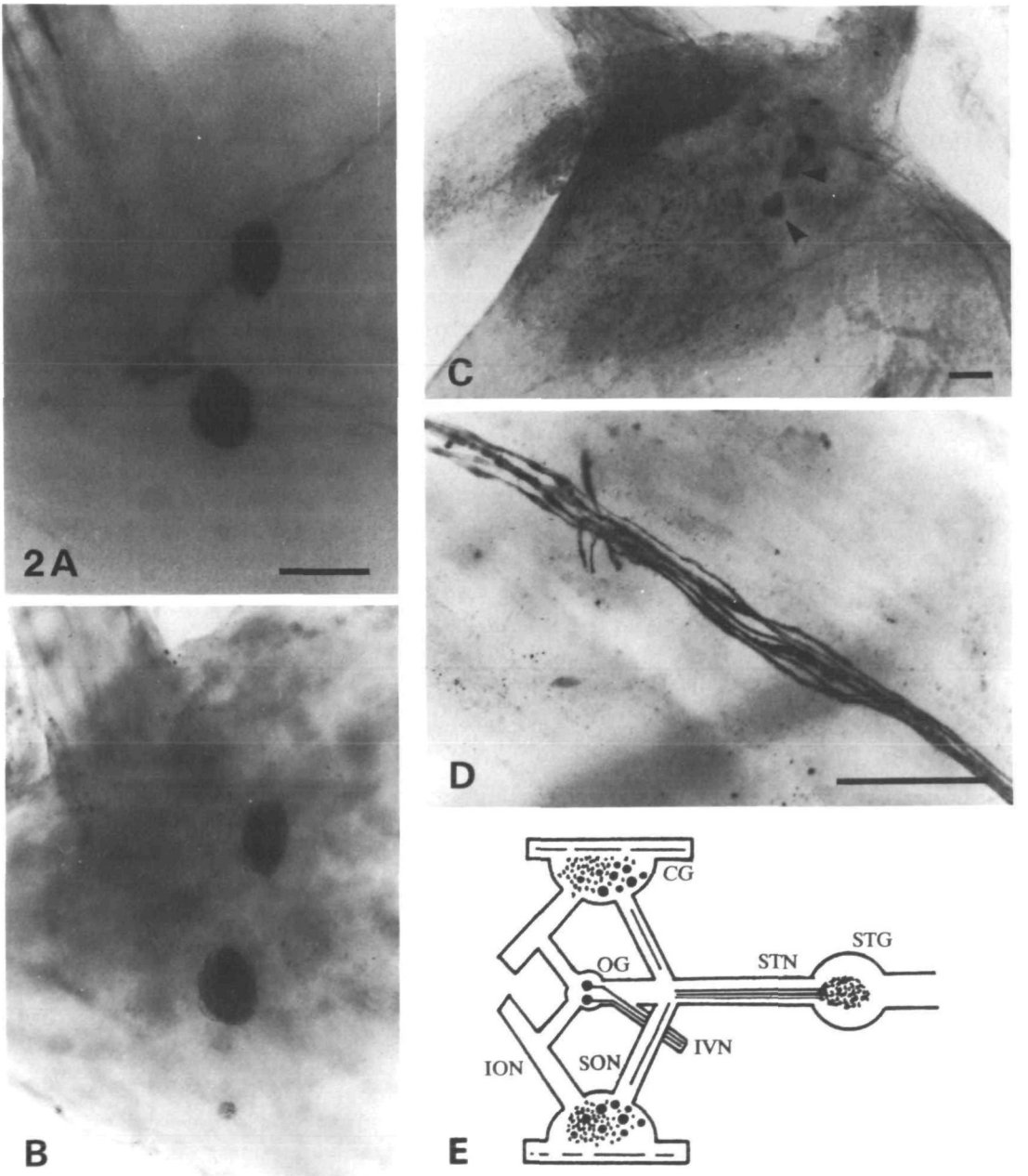
Physiological effects of RPCH on the pyloric rhythm

The pyloric rhythm of the stomatogastric ganglion consists of a repeating, alternating pattern of dilation and constriction of the pyloric region of the stomach (Selverston & Moulins, 1987). This rhythm can be easily recorded with extracellular electrodes on the motor nerves, as can be seen in the first trace of Fig. 3A. This is a recording from the lateral ventricular nerve (LVN), and shows the sequential activity of the lateral pyloric (LP) neurone (largest unit in the recording), pyloric (PY)

Fig. 2. RPLI in the oesophageal ganglion (OG) and commissural ganglia (CGs). (A,B) Double-labelling two neurones in the OG by backfilling the inferior ventricular nerve (IVN) with Lucifer Yellow (A) and subsequently immunolabelling the same OG for RPLI, visualized with a rhodamine-conjugated secondary antibody (B). The IVN is seen at the top left, and the inferior oesophageal nerves (IONs) are towards the right. (C) RPLI in the CG, localized to fine neuropilar processes and several somata (arrowheads). The commissure is at the bottom of the photograph, and the ION is on the top left. (D) Several fibres within the commissure exhibit RPLI. The fibres shown here were from the 'ION side' of the CG. (E) Distribution of RPLI within the stomatogastric nervous system. SON, superior oesophageal nerve. Calibration bars, 100 μm.

neurone (smallest unit in the recording) and pyloric dilator (PD) neurone (medium-sized units in the recording).

When the STG is isolated from the OG and CGs some preparations continue to produce pyloric rhythms, while others become quiescent. Fig. 3 illustrates the effects of bath application of increasing concentrations of RPCH on a robust pyloric rhythm in an isolated STG. In this case the pyloric cycle frequency was $0{\cdot}87 \pm 0{\cdot}02$ Hz (mean $\pm$ S.D.) in the control condition. RPCH applications produced a dose-



dependent increase in the frequency of the pyloric rhythm, to $1{\cdot}21 \pm 0{\cdot}02$ Hz in 10^{-6} mol l^{-1} RPCH (these differences were statistically significant at the $P < 0{\cdot}001$ level by two-tailed t-test). Additionally, increasing RPCH concentrations produced increases in the number of LP neurone action potentials per burst and extended the percentage of each cycle taken up by the LP neurone burst. In the example shown in Fig. 3 the LP neurone fired $7{\cdot}8 \pm 0{\cdot}4$ action potentials per burst in control saline and $16{\cdot}8 \pm 0{\cdot}4$ action potentials per burst in 10^{-6} mol l^{-1} RPCH. Again, these differences were significant at the $P < 0{\cdot}001$ level.

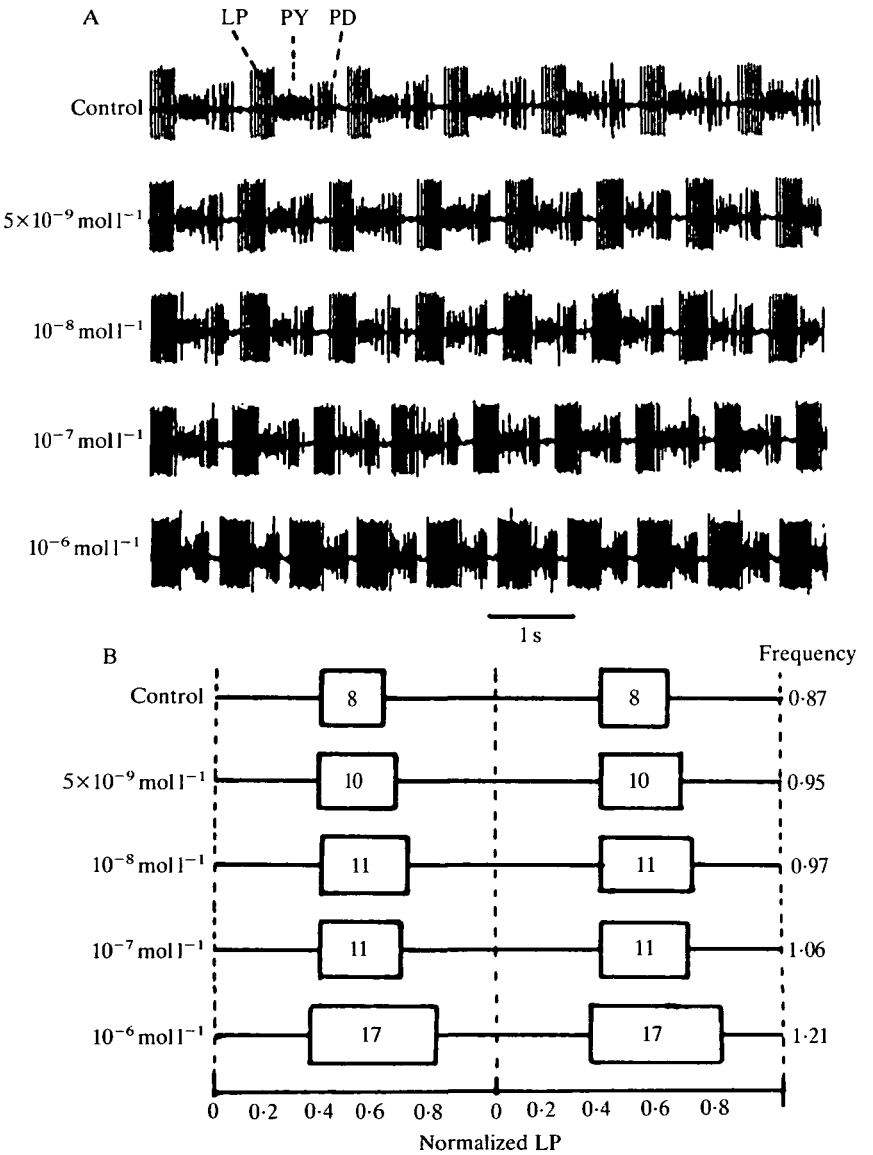


Fig 3

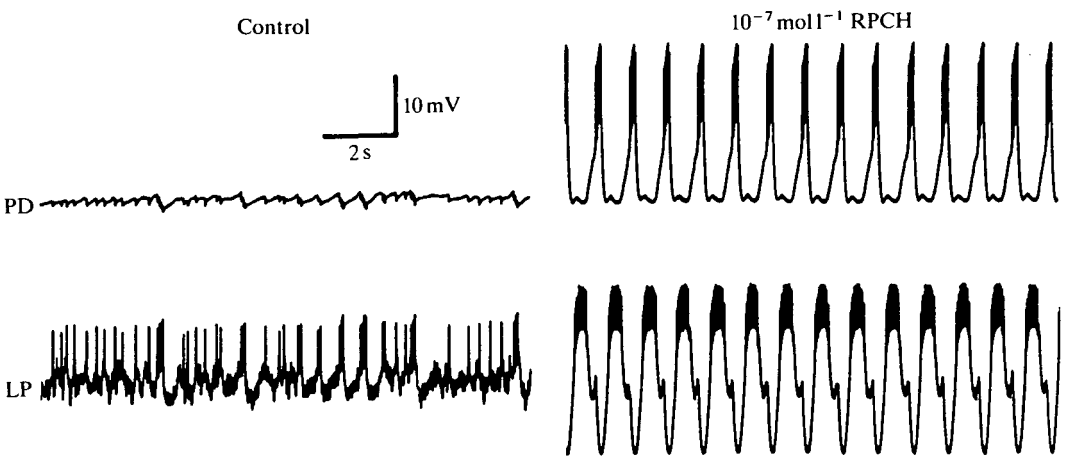


Fig. 4. RPCH activities rhythmic pyloric activity in a quiescent preparation. Left-hand panel: intracellular recordings from pyloric dilator (PD) and lateral pyloric (LP) neurones in saline. Right-hand panel: several minutes after the bath application of 10^{-7} mol l^{-1} RPCH. The effects of RPCH reversed after washing. Baseline membrane potentials were −60 mV (LP) and −58 mV (PD).

RPCH activated rhythmic pyloric cycling when it was applied to quiescent preparations, as shown in recordings from a PD neurone and the LP neurone (Fig. 4). In control saline the PD neurone was silent, whereas the LP neurone fired tonically. After application of 10^{-7} mol l^{-1} RPCH, rhythmic activity commenced, resulting in repeating alternations of bursts of action potentials in the PD and LP neurones.

The threshold for the activation of quiescent preparations by RPCH was around 5×10^{-9} mol l^{-1}. Increases in the concentration of RPCH from threshold to 10^{-6} mol l^{-1} decreased the latency to onset and increased the frequency of the pyloric rhythm (Fig. 5).

The excitatory effects of RPCH on pyloric frequency are summarized in Fig. 6, a plot of the pyloric rhythms recorded in 16 preparations in various RPCH

Fig. 3. Effects of bath-application of RPCH on a spontaneously active pyloric rhythm. (A) Extracellular recordings from the lateral ventricular nerve (LVN) in control saline and in the marked concentrations of RPCH. The preparation was washed for 30 min between each RPCH application, and returned to control levels before RPCH was reapplied. Pyloric frequencies were: control, 0·87 ± 0·02 Hz; 5×10^{-9} mol l^{-1} RPCH, 0·95 Hz; 10^{-8} mol l^{-1} RPCH, 0·97 ± 0·01 Hz, 10^{-7} mol l^{-1} RPCH, 1·06 ± 0·02 Hz; 10^{-6} mol l^{-1} RPCH, 1·21 ± 0·02 Hz. Each of these values differs from each other and from the control levels at the $P<0·001$ level (Student's t-test). (B) The normalized time of firing of the lateral pyloric (LP) neurone is indicated for each concentration of RPCH for the same preparation as seen in A. This shows that the LP neurone extends its impulse burst to later in the cycle in the presence of RPCH. The numbers in the boxes indicate the numbers of LP spikes per burst. For each concentration of RPCH both the number of LP spikes per burst and the normalized end of LP burst are different from the control at $P<0·001$. PY, pyloric neurone; PD, pyloric dilator neurone.

concentrations. The mean control pyloric frequency was $0{\cdot}25 \pm 0{\cdot}36$ Hz (mean $\pm$ S.D., $N = 42$), and the pyloric frequency in 10^{-6} mol l^{-1} RPCH was $1{\cdot}07 \pm 0{\cdot}23$ Hz ($N = 22$). The mean number of LP spikes per burst increased from $3{\cdot}19 \pm 4{\cdot}1$ in saline to $15{\cdot}8 \pm 3{\cdot}8$ in 10^{-6} mol l^{-1} RPCH ($P < 0{\cdot}001$). RPCH increased the number of spikes per burst in another component of the pyloric network, the IC neurone, and caused a slight but consistent decrease in the number of PD spikes per burst.

Comparison of the physiological effects of RPCH and the AKHs

In many preparations RPCH and the AKHs mimic the physiological actions of each other. Therefore, we compared the physiological effects of the AKHs with those of RPCH in six experiments. In the representative recordings shown in Fig. 7, the

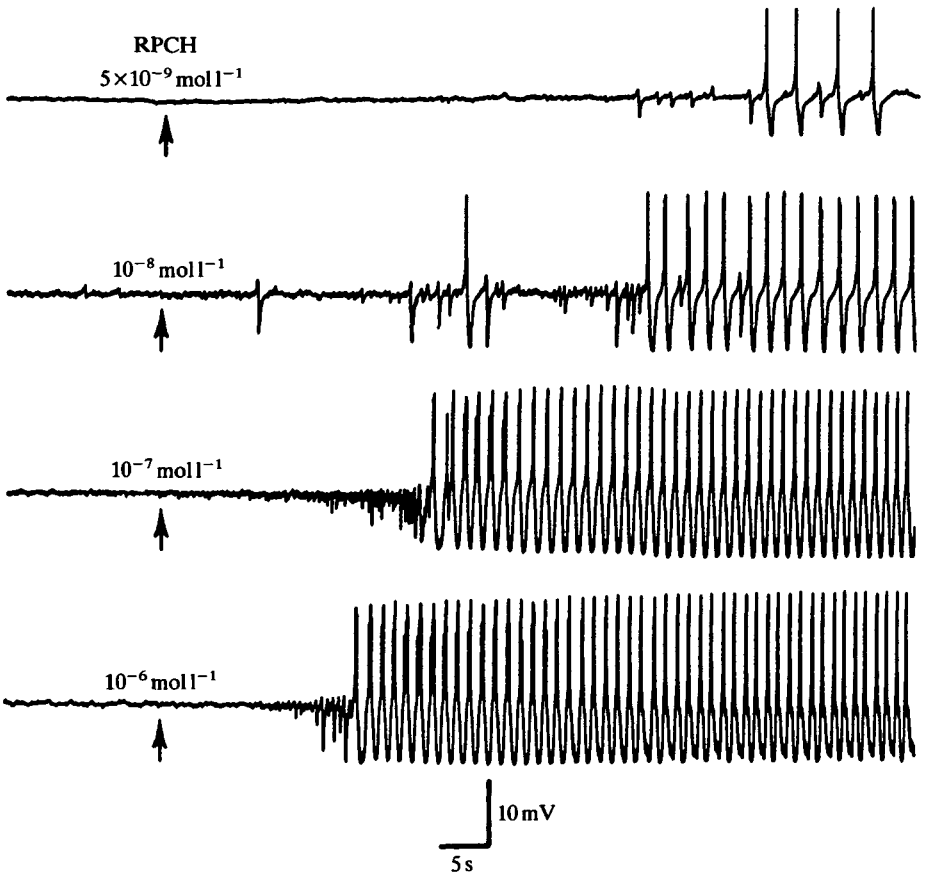


Fig. 5. Dose-dependent activation by RPCH. Intracellular recording from a pyloric dilator neurone (note the slow time base). The arrows show the time at which the designated concentrations of RPCH were applied to the bath. The latency to activation decreased with increasing concentrations of RPCH, and the frequency of the pyloric rhythm increased with increasing concentration. The preparation was washed extensively between applications, and it returned to control quiescent conditions between each application. Baseline membrane potential was -62 mV.

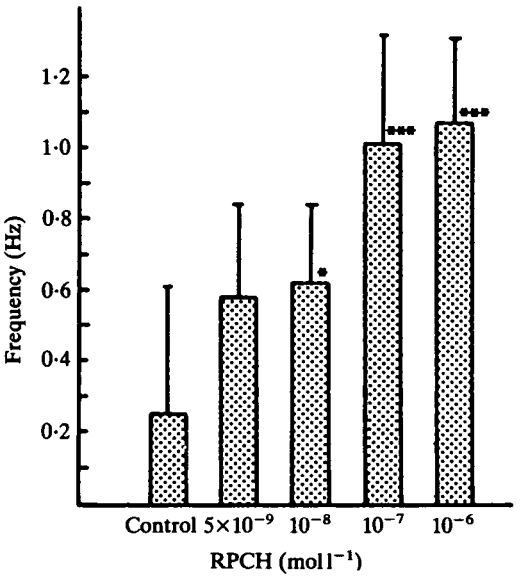


Fig. 6. Effect of RPCH on pyloric frequency. Pooled data from 16 preparations. Preparations showing no rhythmic activity were considered as 0 Hz for these calculations. Controls, taken before each RPCH application ($N = 42$), $0{\cdot}25 \pm 0{\cdot}36$ Hz; 5×10^{-9} mol l^{-1} RPCH ($N = 4$), $0{\cdot}58 \pm 0{\cdot}26$ Hz; 10^{-8} mol l^{-1} RPCH ($N = 5$), $0{\cdot}62 \pm 0{\cdot}22$ Hz; 10^{-7} mol l^{-1} RPCH ($N = 11$), $1{\cdot}01 \pm 0{\cdot}3$ Hz; 10^{-6} mol l^{-1} RPCH ($N = 22$), $1{\cdot}07 \pm 0{\cdot}23$ Hz. * Different from control, $P < 0{\cdot}05$; *** different from control, $P < 0{\cdot}001$.

preparation was virtually silent before peptide application, and returned to quiescence after each wash. RPCH and the AKHs, at 10^{-7} mol l^{-1}, were considerably more potent than were M I and M II. In the same preparation as that shown in Fig. 7, RPCH was the only peptide to evoke a regular repeating pyloric rhythm when applied at 10^{-8} mol l^{-1}.

RPCH effects persist in TTX

There are a number of substances that can initiate rhythmic pyloric activity in the STG, including dopamine, the muscarinic agonist pilocarpine, serotonin, octopamine, proctolin and an FMRFamide-like peptide (Raper, 1979; Anderson, 1980; Marder & Paupardin-Tritsch, 1978; Marder & Eisen, 1984; Flamm & Harris-Warrick, 1986*a*,*b*; Hooper & Marder, 1984, 1987). Several of these, including dopamine and pilocarpine (Raper, 1979; Anderson, 1980; Harris-Warrick & Flamm, 1986), can produce and maintain rhythmic non-impulse-mediated alternations between antagonists in the presence of tetrodotoxin (TTX).

We have found in three experiments that the ability of RPCH to evoke rhythmic alternating patterns of activity is also maintained in the presence of TTX. In the experiment shown in Fig. 8, the preparation was silent after the application of 10^{-6} mol l^{-1} TTX. Soon after 10^{-6} mol l^{-1} RPCH had been applied to the bath, rhythmic alternations of membrane potential ensued in the LP and PD neurones.

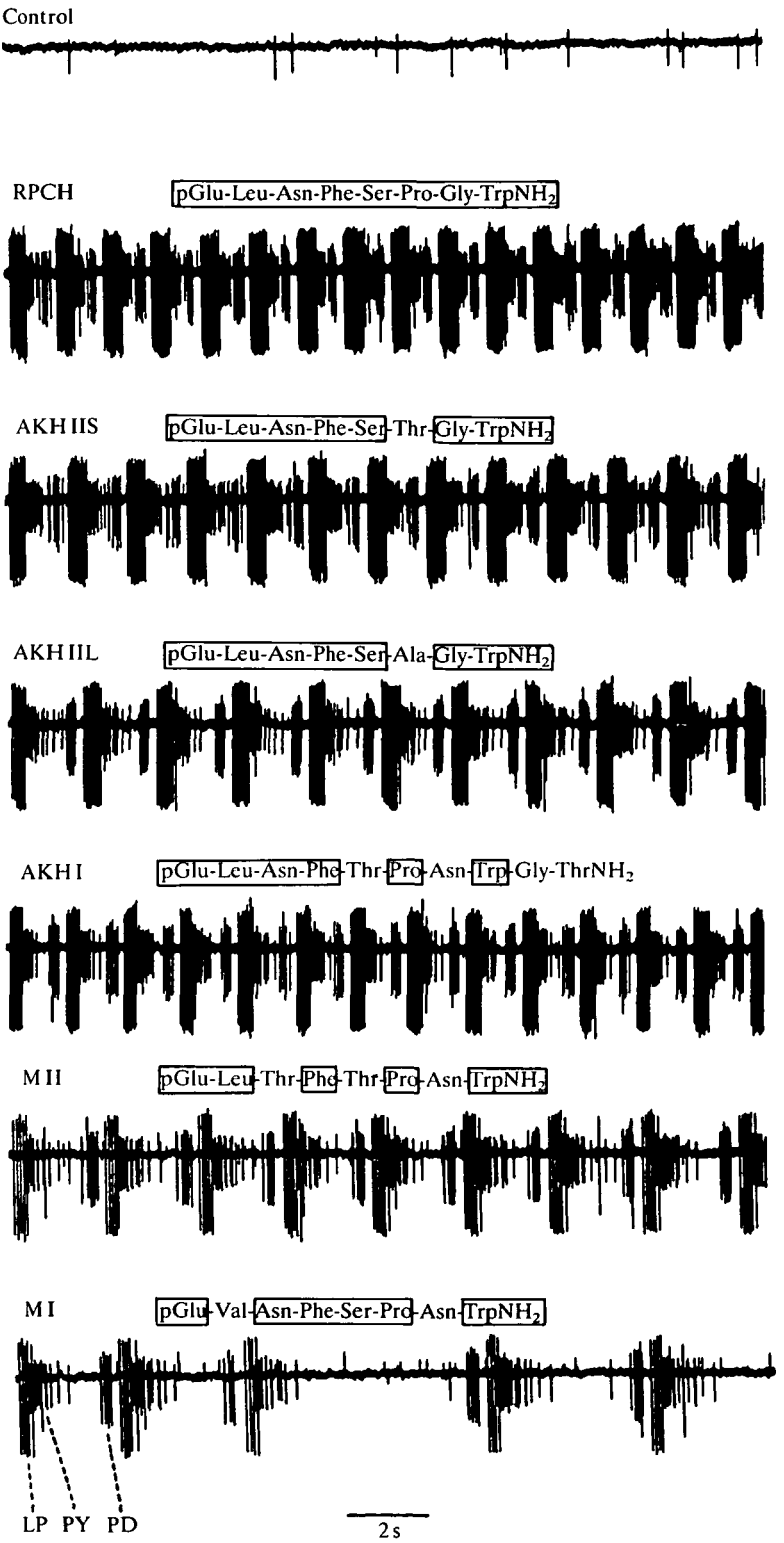

Control
RPCH
pGlu-Leu-Asn-Phe-Ser-Pro-Gly-TrpNH2
AKH IIS
pGlu-Leu-Asn-Phe-Ser-Thr-Gly-TrpNH2
AKH IIL
pGlu-Leu-Asn-Phe-Ser-Ala-Gly-TrpNH2
AKH I
pGlu-Leu-Asn-Phe-Thr-Pro-Asn-Trp-Gly-ThrNH2
M II
pGlu-Leu-Thr-Phe-Thr-Pro-Asn-TrpNH2
M I
pGlu-Val-Asn-Phe-Ser-Pro-Asn-TrpNH2
LP
PY
PD
2s

Fig. 7

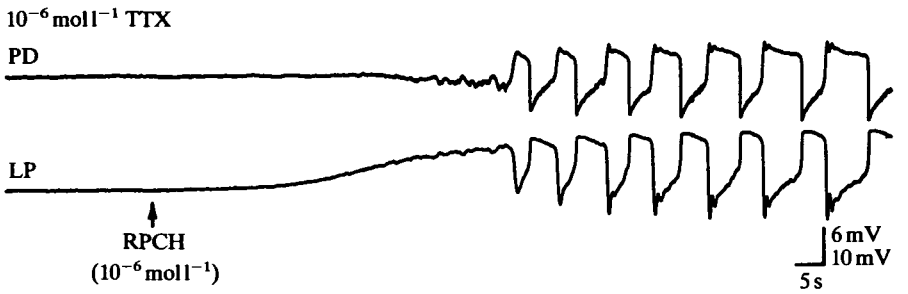


Fig. 8. RPCH activation persists in tetrodotoxin (TTX). Bath application of 10^{-6} mol l^{-1} RPCH to an isolated stomatogastric ganglion continuously perfused with 10^{-6} mol l^{-1} TTX initiated repeating, non-impulse-mediated alternations in membrane potential in the pyloric dilator (PD) and lateral pyloric (LP) neurones. Baseline membrane potentials were −55 mV (PD) and −50 mV (LP).

The frequency of this rhythmic activity, however, was considerably slower than the pyloric frequency that occurs in similar concentrations of RPCH in normal saline. The alternating LP and PD activity persisted as long as RPCH remained in the bath.

DISCUSSION

We have shown that RPCH and the AKHs can produce dramatic physiological actions on the stomatogastric ganglion. These data, together with the finding that RPCH or a closely related peptide is present in input fibres to the STG, provide strong evidence that RPCH is likely to have transmitter/modulator functions in the crustacean nervous system, in addition to its long-known hormonal roles. Thus, RPCH joins a growing list of compounds, such as serotonin, octopamine, dopamine and proctolin, that are thought to be released both into the general circulation from neurohaemal organs in arthropods and to be neurally released into ganglionic neuropiles (Beltz & Kravitz, 1986).

RPCH also joins a growing list of neuromodulatory inputs to the STG that modify the pyloric rhythm (Marder, 1987). Despite the large number of known modulatory inputs to the STG, it appears that each of these produces different effects on the motor patterns of the STG (Marder, 1984, 1987; Marder *et al.* 1987*b*). In this context it is interesting to compare the effects of RPCH with those of the previously described modulatory inputs to the STG. RPCH is most similar in its actions to

Fig. 7. Comparison of the physiological actions of the RPCH/AKH family of peptides. Extracellular recordings from the LVN showing the activity of the lateral pyloric (LP), pyloric (PY) and pyloric dilator (PD) neurones. In control saline (top trace) the preparation was almost completely silent. All peptides were applied at 10^{-7} mol l^{-1}, and the preparation was washed extensively between each application. The amino acid sequence for each peptide is indicated above the response to each peptide. Regions of homology to RPCH are indicated by the closed-in boxes.

proctolin (Hooper & Marder, 1984; Marder *et al.* 1986). RPCH and proctolin can both initiate rhythmic activity in quiescent preparations. Additionally, both substances produce increases in the number of LP neurone spikes per burst. However, in *C. borealis* proctolin also increases the number of PD neurone spikes per burst (M. P. Nusbaum & E. Marder, unpublished results), while RPCH produces a slight decrease in the number of PD neurone spikes per burst. Thus, again, each additional neuromodulatory substance appears to evoke a slightly different variation of the motor patterns produced by the STG.

It is premature to speculate on the nature of the synaptic contacts made by the RPLI-containing terminals in the neuropile of the STG. It is possible that the RPLI fibres make defined synaptic contacts on a subset of the STG neurones. Alternatively, the RPLI-containing terminals may release peptide generally into the neuropile, as a local neurohormone (Jan & Jan, 1982; Sigvardt, Rothman, Brown & Mayeri, 1986). The source of the RPLI-containing fibres that project into the STG is also not yet clear. Possibly these fibres descend from CG somata. It is also possible that they come from somata in the brain or thoracic ganglia *via* some of the RPLI fibres seen in the commissures. .

RPLI is co-localized in two OG somata that also contain an FMRFamide-like peptide (Marder *et al.* 1987*a*) and an SCP_b-like peptide (Callaway *et al.* 1987). These neurones might contain a large peptide that is recognized by several antisera, or they might synthesize and release several different neurally active peptides. This issue must wait for resolution until the protein precursors for these peptides are isolated and purified.

The results of our preabsorption controls suggest that the antiserum of Madsen *et al.* (1985), raised against RPCH, recognizes the amidated tryptophan (-$TrpNH_2$) at the -COOH terminus of RPCH. Of the RPCH/AKH peptides that were tested, the one that was least able to preabsorb the antiserum against RPCH was AKH I. AKH I was also the only tested member of this group that has an amidated threonine instead of an amidated tryptophan at the -COOH terminus (see Fig. 7). At the -NH_2 terminus, the sequences of the first four amino acids are identical in RPCH and AKH I. The fact that M I and M II are 10 times less effective than RPCH and the AKH IIs in preabsorption controls suggests further that the tested antiserum prefers -Gly-$TrpNH_2$ to -Asn-$TrpNH_2$.

For several reasons, our data are consistent with the RPLI in the stomatogastric nervous system being either 'true' RPCH or a very closely related molecule, such as AKH IIS or AKH IIL. First, RPCH, AKH IIS and AKH IIL appear to be similarly effective in activating the pyloric rhythm. Second, although we obtained staining with anti-RPCH antiserum, we saw no AKH-like staining with several different anti-AKH antibodies (M. P. Nusbaum & E. Marder, unpublished results). Third, the peptides that resemble RPCH most closely were most effective in blocking staining. AKH I, while being a potent physiological agonist, was ineffective in blocking RPLI. Fourth, although RPCH has not yet been isolated and sequenced from *Cancer borealis*, it has been characterized biochemically from several different crustaceans (Fernlund & Josefsson, 1972; Carlsen *et al.* 1976; Newcomb, 1983; Mangerich *et al.*

1986), and in each case the peptides are closely related. Fifth, extracts of *Cancer borealis* nervous systems showed RPCH-like bioactivity (K. R. Rao, personal communication). Sixth, injection of purified RPCH produced concentration of the red pigments in *Cancer borealis*, suggesting that the RPCH-like hormonal activity in *C. borealis* is produced by a peptide similar in structure to authentic RPCH (M. P. Nusbaum & E. Marder, unpublished results).

The sinus gland appears to be a major source of hormonally released RPCH. In the crayfish, the RPCH release from the sinus gland is circadian, and is maximal at night (Arechiga, Cortes, Garcia & Rodriguez-Sosa, 1985). For crabs with calcified exoskeletons, the role of RPCH in the control of body pigment is not obvious. However, RPCH also causes pigment concentration in the ommatidia, thus enhancing visual sensitivity (Kulkarni & Fingerman, 1986). This enhanced visual sensitivity might increase foraging success, insofar as many crustacean species are nocturnally active and forage at night. If RPCH is released into the crab haemolymph at high enough concentrations to affect the STG (which is located within the dorsal aorta), then hormonally released RPCH might act to coordinate the animal's feeding behaviour. Alternatively, haemolymph RPCH levels may remain below the threshold for effects on the STG, but may act as a primer for neurally released RPCH by lowering the threshold for neural action, as has been suggested for the effects of serotonin on swimming in the leech (Willard, 1981; Nusbaum & Kristan, 1986).

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