The Journal of Experimental Biology **198**, 1447–1454 (1995)
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MODULATION OF CRAYFISH RETINAL FUNCTION BY RED PIGMENT CONCENTRATING HORMONE

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Accepted 17 March 1995

Summary

The role of the crustacean octapeptide red pigment concentrating hormone (RPCH) in the control of crayfish retinal activity was explored. RPCH injection into intact animals resulted, after a latency of 10–30 min, in a dose-dependent enhancement of electroretinogram (ERG) amplitude lasting 60–120 min.

RPCH was able to potentiate ERG amplitude in both light-adapted and dark-adapted animals. Following light-adaptation, responsiveness to RPCH was five times higher than following dark-adaptation.

In conjunction with ERG enhancement, in light-adapted animals, RPCH injection elicited a dose-dependent retraction of distal retinal pigment, but did not affect proximal retinal pigment position.

The effects of RPCH were blocked by a polyclonal antibody raised against a tyrosinated form of RPCH (A-tyr-RPCH). The antibody was also capable of partially blocking the nocturnal phase of the circadian rhythm of ERG amplitude and the darkness-induced retraction of distal retinal pigment.

These results suggest that RPCH acts both on the retinal photoreceptors and on the distal pigment cells, playing a physiological role as a mediator of the effects induced by darkness and by the nocturnal phase of the circadian rhythm.

Key words: RPCH, neurohormone, crayfish *Procambarus clarkii*, neuropeptide, crustacean, retina, electroretinogram.

Introduction

The response to light in the crustacean compound eye is modulated in a variety of ways. Photoreceptor gain is influenced by efferent pathways. 5-Hydroxytryptamine (5-HT)-like immunopositive axons have been identified close to photoreceptor endings in the crayfish lamina ganglionaris, and 5-HT has been shown to enhance a light-dependent conductance in retinal photoreceptors (Aréchiga *et al.* 1990). The photon flux to the receptors is also a function of the position of pigment granules located inside two sets of cells in the compound eye (see Fig. 1): the proximal pigment, located within the photoreceptor cells, and the distal pigment, located inside long slender cells running from the corneal end of the cell to the basal membrane, parallel to the photoreceptors. These pigments act as filters, regulating the amount of light reaching the retina (Rodríguez-Sosa and Aréchiga, 1982; Shaw and Stowe, 1982; Aréchiga *et al.* 1993). In darkness, both sets of pigments are retracted, leaving most of the photoreceptor surface exposed to light. Under illumination, both are dispersed, to an extent dependent on light intensity, thus blocking the access of stray light to the photosensitive membranes (rhabdoms) in the photoreceptors (see Fig. 1).

The mechanisms by which light- and dark-adaptation elicit the corresponding pigment translocations are different. Whereas proximal pigment migrations are a direct response of the photoreceptors to light and darkness (Olivo and Larssen, 1978; Frixione *et al.* 1979), the distal pigment cells do not respond directly to light; they are the effectors of a neuroendocrine reflex. Distal pigment dispersion is triggered by light acting on extra-retinal photoreceptors (Cortés and Aréchiga, 1984; Aréchiga *et al.* 1985) and it is mediated by the release of an octadecapeptide, the pigment dispersing hormone (PDH) (Fernlund, 1976), which elicits the longitudinal migration of the distal pigment granules. No conclusive evidence is available on the mechanism(s) by which distal pigment retraction is induced.

Another modulatory influence is exerted by a circadian rhythm. At night, the responsiveness of the retinal photoreceptors is much higher than during the day, and both

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retinal pigments are retracted at night and dispersed during the day. This rhythmicity persists under conditions of constant illumination (see Aréchiga *et al.* 1993). The circadian rhythm has been shown to change the retinal responsiveness by 100-fold (Rodríguez-Sosa and Aréchiga, 1982), which is a range similar to that covered by the dynamic range of the response/log intensity function of crayfish retinal photoreceptors (Glantz, 1968).

A new possibility for efferent control of retinal activity is suggested by the characterization of axons immunopositive to an antibody raised against the red pigment concentrating hormone (RPCH). This octapeptide (Fernlund and Josefsson, 1972) is known to promote the radial retraction of pigment granules within tegumentary chromatophores (see Rao, 1985). A host of anti-RPCH immunopositive neurones have been identified in the crayfish eyestalk (Mangerich *et al.* 1986; Bellon-Humbert *et al.* 1986), and recently some immunopositive axons have been traced to the lamina ganglionaris, in a region close to the ending of the photoreceptor axons and to the proximal end of the distal pigment cells (Preciado *et al.* 1994). The injection of RPCH has been reported to enhance electroretinogram amplitude in the crayfish *Orconectes limosus* (Gaus and Stieve, 1992). These observations suggest a physiological role for RPCH in the modulation of crayfish retinal sensitivity, and it is the purpose of this study to present evidence supporting this possibility.

Materials and methods

The experiments were conducted using adult specimens of the crayfish *Procambarus clarkii* Girard of both sexes and during intermoult at the time of the experiments. In total, 315 crayfish were used. The animals were tethered to a recording chamber, containing a low level of water (approximately 5 cm) to ensure humidification of the gills. The ERG was recorded in the conventional way, with a polished insulated stainless-steel needle, 5 μm in diameter at the tip and with a resistance of about 0.1 MΩ. It was inserted beneath the chitinous exoskeleton at the rim of the compound eye and glued to the exoskeleton to produce stable recordings. Signals were preamplified and filtered with a 10 kHz optimum band-pass filter (66.6 % suppression of signal amplitude at frequencies of 5 and 15 kHz). A low-frequency cut-off filter was set at 60 Hz. Recordings were stored on a tape recorder or as print-outs using a Grass Polygraph model 7.

Light pulses were delivered from an incandescent white light source. Light intensity was regulated with neutral density filters (Kodak, Wratten no. 96) and was calibrated using a photometer (Gossen Lunasix 3). Pulse duration was regulated using an electromechanical shutter. Stimulation conditions were adjusted to give a response amplitude with a minimum signal-to-noise ratio of 2:1 for a noise amplitude of approximately 10–20 μV. However, the signal-to-noise ratio was commonly over 10:1.

Animals were injected through a cannula inserted under the carapace and glued to it. The dorsal region of the cephalothorax was most commonly used for cannula implants. RPCH was dissolved in saline solution (van Harreveld, 1936) and 100 μl injections were given. RPCH concentrations given below are those corresponding to the concentrations of the injected solutions.

The positions of the proximal and distal retinal pigments were determined micrometrically. Whole eyestalks were removed at the end of the experimental period and dipped into boiling water for 3–5 min, for instant fixation. They were then transferred to 10 % formaldehyde for at least 24 h before sectioning. The positions of the accessory pigments were determined by direct observation under the microscope of bissected eyestalks or retinas or of thick (100 μm) sections. Pigment position was expressed, following convention, as the proximal pigment index (PPI) and distal pigment index (DPI) (see Fig. 1 for an explanation of how DPI and PPI are calculated). For both pigments, these index values theoretically range from 0 to 1, for complete retraction to full dispersion, respectively. In fact, full distal pigment dispersion was rarely seen. In most experiments, the position of the shielding pigments was stable after 1 h of either light- or dark-adaptation.

RPCH was purchased from Peninsula Laboratories (611 Taylor Way, Belmont, CA 94002-9914, USA). The anti-RPCH antibody (raised against A-tyr-RPCH) was produced in our laboratory and it was the same batch of serum previously used for immunocytochemistry and ELISA determinations (Rodríguez-Sosa *et al.* 1994*a*). This antibody recognizes an epitope with the sequence of the last five residues (Phe-Ser-Pro-Gly-Trp) near the 'amido' terminal of the RPCH molecule.

Amplitude, phase and period length of circadian ERG rhythm were determined in the conventional way (see Rodríguez-Sosa *et al.* 1994*b*).

Results

Effect of RPCH on ERG amplitude

In intact animals (N=21), RPCH was injected into the haemolymph through a cannula implanted under the carapace. The animals were subjected to a background illumination of 175 lx for 1 h, with additional test light pulses of 350 lx and 200 ms duration applied at regular intervals of 3.8 min. As shown in Fig. 2A, 10 min after the injection of 100 μl of 4×10^{-7} mol l^{-1} RPCH, there was a noticeable enhancement of ERG amplitude, which reached twice the control value. The effect was fully established after 25 min and lasted for over 1 h. The magnitude of the effect and its duration were proportional to the dose of RPCH. Fig. 2B illustrates the dose–response function of RPCH on ERG amplitude. The curve is sigmoidal in shape, with a dynamic range from 5×10^{-8} to 10^{-6} mol l^{-1}.

As described above, ERG enhancement may be due to a direct effect of RPCH on the photoreceptors or to an indirect effect through changes in the position of the retinal shielding pigments. At a background illumination of 175 lx, the distal pigment should be fully retracted, whereas the proximal retinal pigment should be intermediate between retraction and

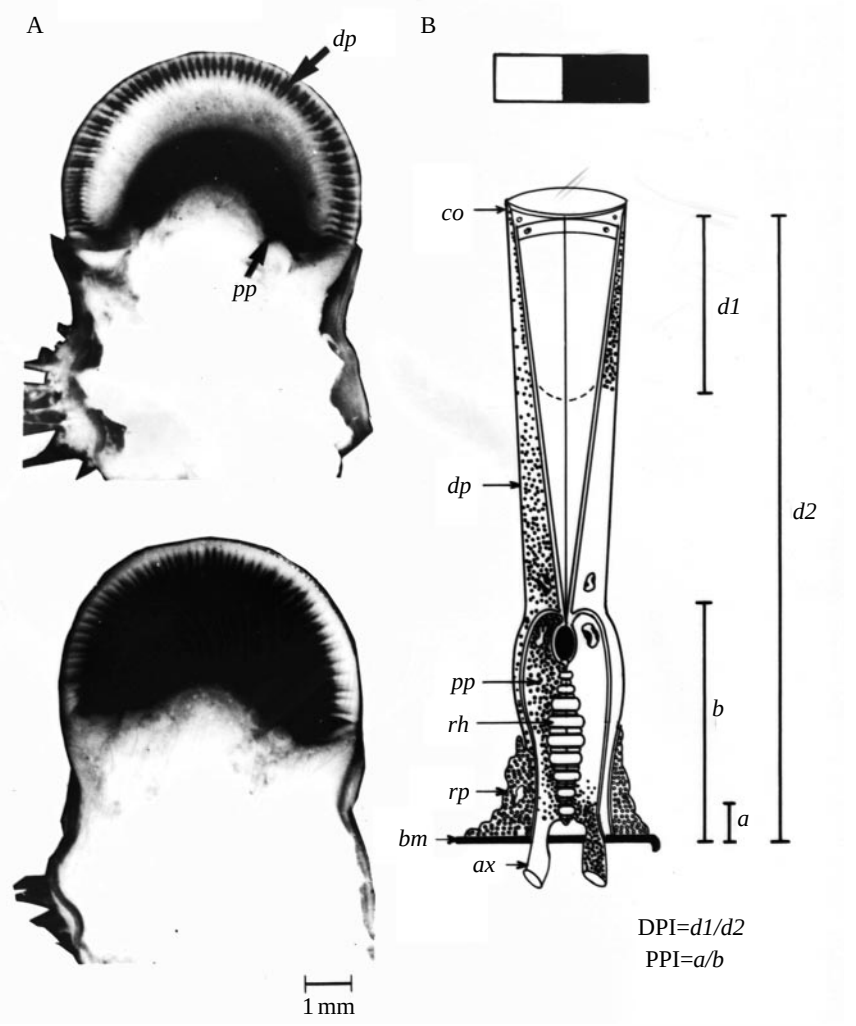

Fig. 1. (A) Bisected crayfish eyestalks showing the position of retinal shielding pigments as two dark layers. In dark-adapted animals (upper photograph), the retinal pigments are retracted, the distal pigment (*dp*) towards the corneal end of the compound eye and the proximal pigment (*pp*) towards the photoreceptor axons in the proximal part of the retina. After light-adaptation (lower photomicrograph), both pigments are dispersed, shielding the photoreceptor area. (B) Diagrammatic representation of the cellular elements in the ommatidia and the micrometrical references used to quantify pigment position. *co*, corneal cells; *dp*, distal pigment; *pp*, proximal pigment; *rh*, rhabdom; *rp*, reflecting pigment; *bm*, basal membrane; *ax*, photoreceptor axons; *d1*, distance from the corneal cells to the proximal end of the distal pigment; *d2*, distance from the corneal cells to the basal membrane; *a*, distance from the distal end of the proximal pigment to the basal membrane; *b*, distance from the distal end of the photoreceptors to the basal membrane. DPI, distal pigment index; PPI, proximal pigment index. Open and filled bars at the top of B indicate light- and dark-adaptation, respectively, in the main diagram.

dispersion (Aréchiga, 1977; Fernández de Miguel and Aréchiga, 1992). In order to investigate the possible direct effect of RPCH on photoreceptors, the effect of RPCH injection was tested on animals previously dark-adapted for 2 h. The potentiating effect of 4×10^{-7} mol l^{-1} RPCH on ERG amplitude was present under these experimental condition (Fig. 3A), while both pigments remained retracted throughout the experiment. The positions of both pigments were also assessed in control animals subjected to the same stimulation protocol, but injected with saline solution alone. ERG potentiation elicited by RPCH in dark-adapted animals was also dose-dependent but, as seen in Fig. 3B, the threshold dose was five times higher than that for light-adapted animals; the dynamic range of the dose–response function was from 10^{-7} to 2×10^{-6} mol l^{-1}.

Effect of RPCH on retinal pigment position

From the previous experiment, it seems clear that the effect of RPCH on ERG amplitude occurs even when the retinal shielding pigments are retracted, but it is possible that in the light-adapted animals, in which retinal pigments are partly or fully dispersed, pigment retraction may contribute to the effect of RPCH on ERG amplitude. To test this possibility, RPCH was injected into light-adapted animals in which the proximal pigment was fully dispersed and the distal pigment was partly dispersed. Four groups of animals (N=21 for each group) were kept under a background illumination of 5000 lx for 180 min prior to injection and killed at 10 min intervals after injection of either saline (control) or RPCH at three different concentrations. RPCH elicited a dose-dependent retraction of the distal pigment (Fig. 4). No effects were detected on the proximal pigment distribution. Distal pigment retraction started 10 min after injection, was fully established after 25–30 min and lasted for 1 h. The magnitude of the effect was again dose-dependent, within the range 10^{-8} to 3×10^{-6} mol l^{-1} (Fig. 4B). No effects of RPCH were detected on either set of retinal shielding pigments after retraction induced by dark-adaptation.

Specificity of the effect of RPCH

A method by which to explore the specificity of RPCH actions on the retina is to block them using a specific agent. We therefore tested the A-tyr-RPCH antibody previously raised against a tyrosinated form of RPCH, which has been used to identify and quantify RPCH in the eyestalk (Rodríguez-Sosa *et al.* 1994*a*). This antibody recognizes the last 3–5 residues near the amido

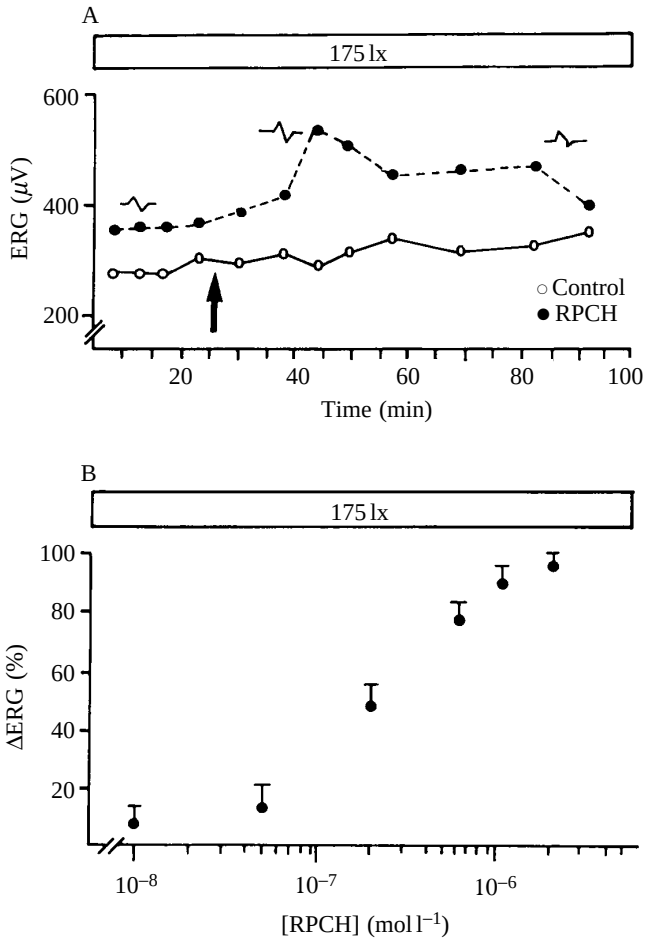

Fig. 2. Effect of RPCH on ERG amplitude in a single crayfish *Procambarus clarkii* adapted to a background light of 175 lx. (A) Effect of a single 100 μl injection of 4×10^{-7} mol l^{-1} RPCH at the point indicated by the arrow (filled circles). Open circles are control values (saline only). Bipolar ERG recordings are shown for three points during the experiment. (B) Dose–response relationship for a group of five individuals. The *y*-axis shows percentage ERG enhancement over the control value. Points indicate mean values for at least three determinations and 1 S.D.

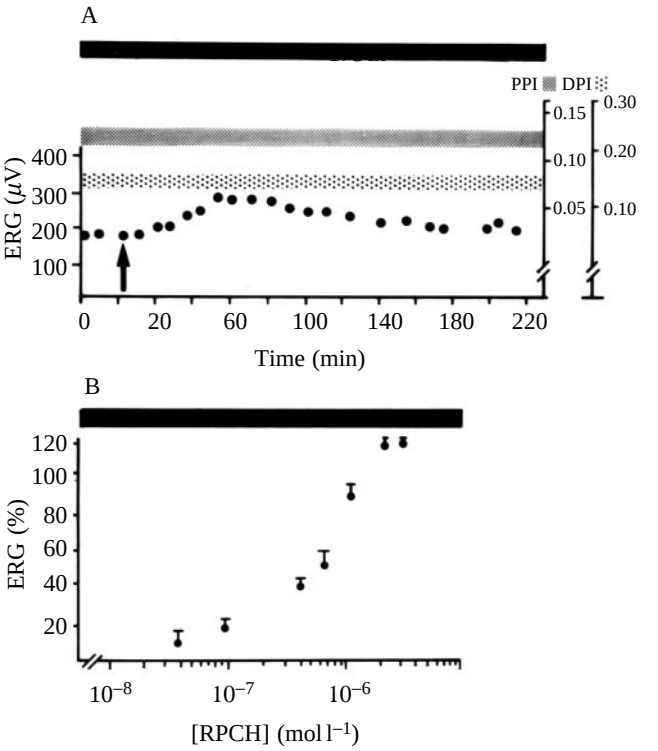

Fig. 3. Effects of RPCH on five dark-adapted *Procambarus clarkii*. Black bars indicate darkness throughout the experiment. (A) Potentiating effect of an injection of 100 μl of 4×10^{-7} mol l^{-1} RPCH on ERG amplitude (filled circles) in a single individual; proximal and distal pigment distributions are unaffected. Proximal pigment index (PPI; hatched bar) and distal pigment index (DPI; stippled bar) are also shown. PPI and DPI values indicate the ranges of pigment positions under the experimental conditions. The arrow shows the time of RPCH injection. (B) Dose–response relationship for the effect of RPCH on ERG amplitude on all five dark-adapted animals. The *y*-axis shows the percentage ERG amplitude enhancement. Each point represents the mean of at least three determinations and 1 S.D.

terminal and, in previous tests, it was found to block the effect of RPCH on the erythrophores in isolated tegumentary flaps. In a group of five animals, the effect of RPCH was explored using the protocol described above to test for the effect of RPCH on the shielding pigments. However, in some animals, the antibody (A-tyr-RPCH) was injected 30 min before RPCH injection, at a dilution of 1:350, a concentration that was found to be effective on the tegumentary chromatophores. As seen in Fig. 5, the effect of 4×10^{-7} mol l^{-1} RPCH on ERG amplitude was almost completely blocked by the presence of the antibody. A-tyr-RPCH was also capable of blocking the RPCH-induced retraction of distal pigment in light-adapted animals (results not shown).

Possible physiological role of RPCH

The above results suggest a physiological role for RPCH in the control of the retraction of distal retinal pigment elicited by darkness and on the nocturnal phase of the circadian rhythm of ERG amplitude. These possibilities were explored by testing whether A-tyr-RPCH antiserum was capable of blocking both phenomena. Two protocols were devised. (a) Two batches of animals (control and A-tyr-RPCH-injected) were kept under a background illumination of 517 lx. Groups of three animals were killed at 1 h intervals during the day. At 13:00–14:00 h, either saline solution or a 1:350 dilution of A-tyr-RPCH was injected. The proximal pigment is fully dispersed during the day, whereas the distal pigment is only half-dispersed (Fig. 6). At dusk in control animals, the distal pigment spontaneously undergoes a partial retraction due to its endogenous circadian rhythm. At the season in which these experiments were conducted (November–December), the retraction started at about 15:00 h. This migration was blocked by injecting a 1:350 dilution of A-tyr-RPCH 30 min before its expected onset. The proximal pigment remained fully dispersed throughout the experiment (Fig. 6). Saline or more

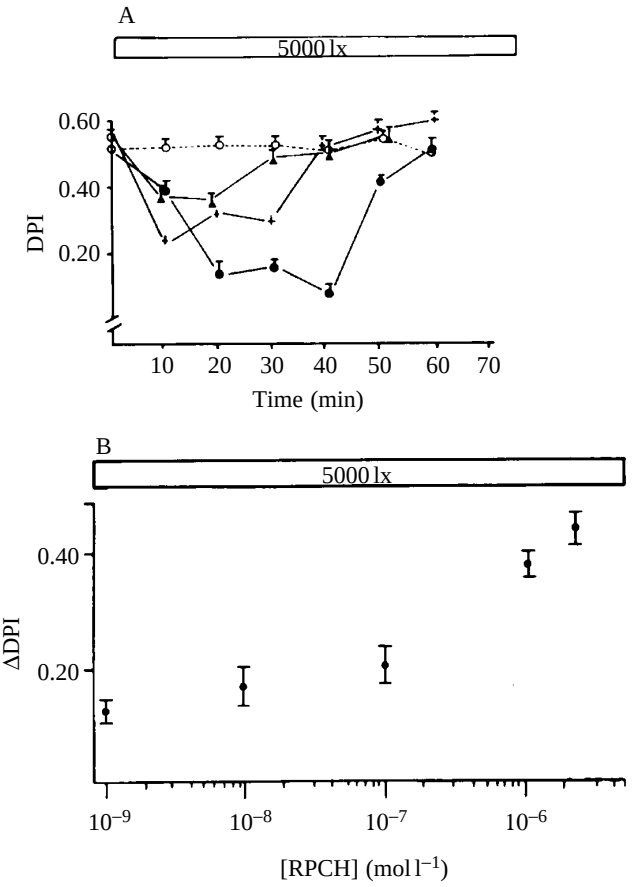

Fig. 4. The effect of RPCH on distal pigment index (DPI) in light-adapted *Procambarus clarkii*. (A) Time course of distal pigment retraction in four groups of animals after saline or RPCH injection at the concentrations shown. Injection was at 0 min, *N*=21 for each group. Open circles, saline; filled triangles, 10^{-8} mol l^{-1} RPCH; crosses, 10^{-7} mol l^{-1} RPCH; filled circles, 10^{-6} mol l^{-1} RPCH. (B) Dose–response relationship for the same animals. The *y*-axis shows the extent of RPCH-induced retraction, in DPI units, above control values. Each point represents an average of at least three determinations ±1 S.D. from the same group of animals.

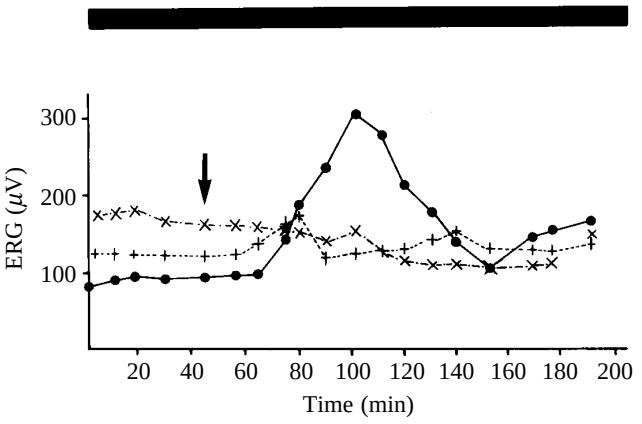

Fig. 5. Blockage of RPCH-induced ERG enhancement by injection of A-tyr-RPCH in dark-adapted *Procambarus clarkii.* Animals were injected with saline (+), 4×10^{-7} mol l^{-1} RPCH (●) or 4×10^{-7} mol l^{-1} RPCH (indicated by the filled arrow) preceded by A-tyr-RPCH injection (×, 30 min earlier, injected at the arrow). Values are for representative responses from 1–5 animals in each of the three series.

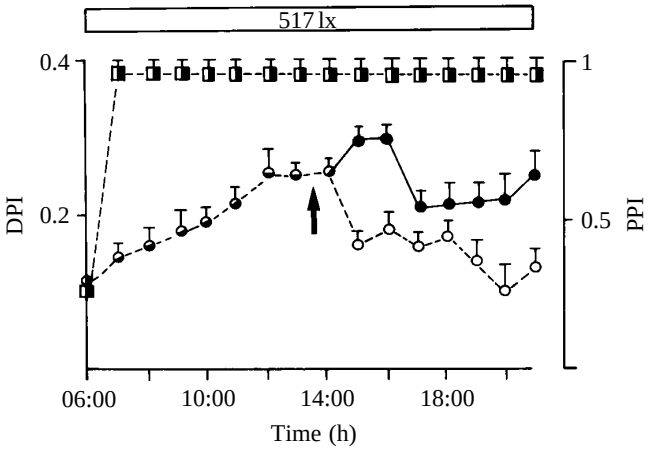

Fig. 6. Blockage of distal pigment retraction at dusk (see text) in light-adapted animals, by injection of a 1:350 dilution of A-tyr-RPCH, without affecting proximal pigment position. Left axis, distal pigment index DPI (circles); right axis, proximal pigment index PPI (squares). Open symbols controls; filled symbols, injected animals. Each point indicates the mean of at least three determinations (from separate groups of animals) and 1 S.D. (*N*=32 animals). A-tyr-RPCH was injected at the arrow.

dilute solutions of A-tyr-RPCH did not prevent distal pigment retraction at dusk. (b) A second method used to investigate the physiological role of RPCH was to record the ERG for 24 h cycles. Animals were kept in darkness, only interrupted by test light pulses of 2 lx and 200 ms duration applied at regular 1 h intervals. Fig. 7 shows 1 week of continuous recording from a dark-adapted animal, on day 4, 1:350 A-tyr-RPCH was injected. A slight reduction of the amplitude of the circadian rhythm and a small lengthening of the circadian period during the first 24 h cycle after A-tyr-RPCH injection were detected. No long-term phase shifts or changes in the length of the circadian period could be detected. No other effects were found during several further days of continuous ERG recording. A-tyr-RPCH was injected at various points during the 24 h cycle in five animals, and in no case was a long-term change of phase or circadian period length detected (results not shown).

Discussion

The enhancement of ERG amplitude induced by RPCH injection in intact animals is in agreement with the effect reported by Gaus and Stieve (1992) in isolated retinas. The effect of RPCH injection on the distribution of the distal retinal pigment, however, suggests that RPCH may indeed be the sought-after dark-adapting hormone for this pigment (see Kulkarni and Fingerman, 1986). Since RPCH enhances ERG amplitude even when both retinal shielding pigments are fully retracted, RPCH probably has a dual action consisting of both a direct action on the photoreceptors, presumably at some stage of the phototransduction cascade, and an indirect effect on the

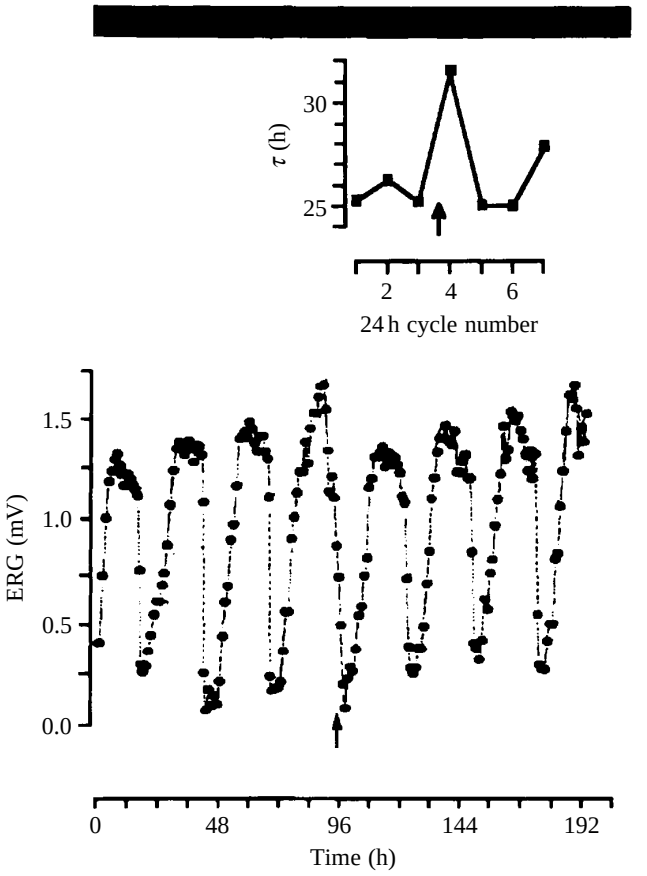

Fig. 7. Lack of a long-term effect of 1:350 A-tyr-RPCH injection (indicated by an arrow) on the amplitude of the circadian ERG rhythm in dark-adapted animals. Inset: the effect of A-tyr-RPCH injection (arrow) on the circadian period (τ).

distal retinal pigment. It has been suggested that 5-hydroxytryptamine (5-HT) acts in parallel on both the retinal phototransduction process and on a retinal accessory pigment, the proximal pigment (Aréchiga *et al.* 1990). Octopamine also appears to have a dual action on *Limulus polyphemus* retinal receptors and pigment cells (see Kass and Barlow, 1984). In fact, parallel synergistic mechanisms of control have been described for photoreceptors and accessory pigments of several invertebrate species (Fleissner and Fleissner, 1988). Direct effects of RPCH on neuronal activity have been documented for the crab stomatogastric ganglion (Nusbaum and Marder, 1988) and the crayfish swimmeret system (Sherff and Mulloney, 1991). No evidence is available as to the possible mechanisms by which these effects are produced.

The dose range required for both effects is higher than that previously reported for RPCH action on tegumentary chromatophores (see Rao, 1985). However, it is worth considering that the concentrations given here are those of the injected solutions and that 100-fold dilution is expected to occur in the circulation (Riegel and Parker, 1960). Owing to permeability barriers, access to the target cells in the retina is also presumably lower than to the tegumentary chromatophores. A similar mismatch was reported for the effects of 5-HT on the retina when compared with its effects on muscles (Aréchiga *et al.* 1990). There may also be a difference in sensitivity of the respective cellular receptors. There is no explanation currently available to account for the observed differences between the threshold doses required for the effects of RPCH on light-adapted and dark-adapted animals. Perhaps the lower sensitivity of the latter was due to the fact that, in darkness, RPCH levels are spontaneously higher than in light-adapted animals.

The lack of an effect of RPCH on the proximal retinal pigment indicates the presence of a different target from that reported for 5-HT, which induces proximal pigment retraction while also acting directly on the photoreceptors (in this case, by activating a light-induced conductance; Aréchiga *et al.* 1990). The actual site(s) of RPCH action appears to be in the retina itself, given the similarity of our results to those obtained by Gaus and Stieve (1992) on the ERGs of isolated *Orconectes limosus* retinas, as well as the presence of RPCH-like immunopositive axons in the base of the retina (Preciado *et al.* 1994). However, intermediate sites of RPCH action cannot be ruled out from our results.

The blockage by A-tyr-RPCH of darkness-induced distal pigment retraction and of the nocturnal rise in ERG amplitude in dark-adapted animals suggests that RPCH is a physiological mediator of these phenomena. The RPCH content in the crayfish eyestalk is more than two orders of magnitude higher at night than during the day (Rodríguez-Sosa *et al.* 1994*b*). The RPCH-like immunopositive neurones in the medulla externa, from which axons lead to the lamina ganglionaris, in the regions where the photoreceptor axons end, are likely to be the same neurones for which Kirk *et al.* (1983) reported tonic activity under darkness and inhibition elicited by illumination. Presumably, under physiological conditions, RPCH reaches the retinal cells by two pathways, one of them blood-borne, since the highest content of RPCH in the crayfish eyestalk is in the sinus gland, a neurohaemal organ, from which it is released to the haemolymph (Rodríguez-Sosa *et al.* 1994*a*). RPCH release from the sinus gland by direct electrical stimulation is higher at night than during the day (Aréchiga *et al.* 1985). The other pathway would be a local release from the endings of the RPCH-containing neurones in the medulla externa. It is, as yet, unclear whether these two pathways might play different physiological roles in the retina.

RPCH is unlikely to be the only mediator of the effects of darkness or circadian activity on retinal cells. For example, as discussed above, 5-HT is also likely to have an effect; its content in the eyestalk is higher at night (Fingerman and Fingerman, 1977). These mediators might exert complementary actions in the nocturnal facilitation of retinal activity in the crayfish.

The dispersion of distal retinal pigment under illumination or during the day is likely to be mediated by the pigment dispersing hormone (PDH), an octadecapeptide (Fernlund, 1976) that is also stored in the sinus gland and released by light acting on extra-retinal photoreceptors (Aréchiga *et al.* 1985). PDH content in the crayfish eyestalk is higher during the day

than at night (Aréchiga and Mena, 1975). PDH-like immunopositive neurones have also been identified in the lamina ganglionaris (Mangerich *et al*. 1987), in the region where the RPCH-like immunopositive neurones branch.

It is, therefore, a plausible assumption that the photomechanical responses of the distal pigment are mediated by two neuroendocrine reflexes. The first, triggered by light, presumably acts through extra-retinal photoreceptors (Cortés and Aréchiga, 1984), resulting in the release of PDH and promoting distal pigment dispersion. The second reflex would be triggered by darkness and mediated by RPCH release, resulting in retraction of the distal pigment. The observed circadian rhythm of distal pigment migration would then be a consequence of the circadian rhythmicity of PDH and RPCH secretion. More detailed studies are necessary to validate this hypothesis.

From the results reported here, the effects of RPCH on the circadian rhythm of ERG amplitude appear to be of a modulatory nature on the retinal effectors of the rhythm. Since no phase shifts could be detected in the circadian rhythm of ERG amplitude, a direct action on the site of generation of circadian rhythmicity seems unlikely.

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