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MODULATION OF SCREENING-PIGMENT POSITION IN CRAYFISH PHOTORECEPTORS BY SEROTONIN: POSSIBLE INVOLVEMENT OF Na^+/K^+-ATPase ACTIVITY

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Summary

The possibility that serotonin might play a role in the modulation of screening-pigment position in crayfish photoreceptors was explored through experiments with isolated eyes and a membrane fraction from retinal homogenates. In the isolated eye serotonin ($\geq 10^{-4}$ mol l^{-1}) and some of its agonists exerted a limited dark-adapting influence over the pigment position, irrespective of the presence or absence of light, and this effect was abolished by the simultaneous addition of serotonin antagonists. In the retinal membrane fraction serotonin and quipazine produced a methysergide-sensitive stimulation of the Na^+/K^+-ATPase activity. These results are interpreted in terms of a serotonin-mediated efferent input on the photoreceptors, which would affect the ionic regulation of the pigment transport mechanisms.

Introduction

The migrations of screening-pigment granules within the photoreceptor cells of compound eyes have long been recognized as a major feature in the visual physiology of arthropods (see Parker, 1932). Shifts in pigment position can influence the absolute and spectral sensitivities of the photoreceptors, as well as their angle of acceptance for incident light rays and, therefore, the spatial resolution of the retina (for reviews see Miller, 1979; Stavenga, 1979; Rao, 1985). In the past decade several studies have investigated the mechanisms involved in both the translocation of the pigment particles (Miller, 1975; Frixione *et al.* 1979; Frixione, 1983*a*,*b*) and the coupling of photoreceptor excitation with pigment migration (Olivo & Larsen, 1978; Kirschfeld & Vogt, 1980; Frixione & Aréchiga, 1981; Frixione & Ruiz, 1988). Although the current picture of the processes that mediate the light-dependent migrations of the screening pigment is far from complete, even less is known about the mechanisms that participate in the light-independent pigment movements observed in the photoreceptors of some species.

The occurrence of regular pigment migrations unrelated to illumination in

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arthropod photoreceptors was first documented for crustaceans. Crayfish kept in the dark for several days displayed partial pigment dispersion during the daytime and full pigment aggregation at night (Bennitt, 1932). Although this apparently spontaneous behaviour of the pigment could be ascribed to a circadian rhythmicity of the photoreceptor itself, various observations suggest that it might rather be the result of efferent modulation in which serotonin could play a significant role. First, isolated eyes incubated in constant darkness show the retinal pigments in a fixed position, despite a clear circadian rhythm in the amplitude of the electroretinogram (Sánchez & Fuentes-Pardo, 1977), and even though the excised retinas retain the capability of achieving at least one full cycle of pigment migration – i.e. a complete aggregation followed by a complete dispersion – in response to darkness and illumination (Frixione *et al.* 1979). Second, the levels of serotonin in some crustacean eyestalks undergo circadian variations with a large increase during the evening hours (Fingerman & Fingerman, 1977), when pigment migration to the dark-adapted position normally takes place, and injection of exogenous serotonin into the eyes of a closely related arthropod greatly enhances their sensitivity to light (Barlow *et al.* 1977). Third, serotonin-like immunoreactivity is prominent in three of the four optic ganglia located in crayfish eyestalks (Elofsson, 1983), and can be traced in fibres closely associated with the photoreceptor axons in the first optic ganglion (H. Aréchiga, E. Bañuelos, E. Frixione, A. Picones & L. Rodríguez-Sosa, unpublished results). Fourth, a number of findings point to serotonin as a key wide-spectrum neuromodulator in crustaceans (Beltz & Kravitz, 1986). Fifth, serotonin appears to be a circadian modulator in the visual systems of other invertebrates (Nadakavukaren *et al.* 1986), as well as in those of some vertebrates (Dearry & Burnside, 1986).

In this paper we report experiments with isolated eyes of the crayfish incubated in the presence of serotonin and serotonergic agonists and antagonists, which confirm an influence of this amine upon pigment positioning within the photoreceptors. In addition, these compounds are shown to affect the Na^+/K^+-ATPase activity in a cellular fraction enriched in photoreceptor membranes. The results are discussed in the context of current views on serotonin as a neuromodulator and on the ionic control of screening-pigment position in arthropod photoreceptors.

Materials and methods

Chemicals

Serotonin (5-hydroxytryptamine creatinine sulphate, 5-HT), 5-methoxy-*N*,*N*-dimethyltryptamine (5-MDT), ouabain, pargyline and adenosine 5′-triphosphate (ATP, vanadium-free, catalogue no. A-0270) were purchased from Sigma Chemical Co. (St Louis, MO, USA). Methysergide bimaleate and quipazine maleate were a generous donation of Laboratorios Miles de México, SA. Cyproheptadine hydrochloride was kindly provided by Merck Sharp & Dohme de México, SA.

Animals and experiments with isolated eyes

Adult crayfish of both sexes of the species *Procambarus clarkii* (Girard) were

obtained from specialized collectors and kept in outdoor aquaria under natural illumination. Eyestalks excised from these animals were prepared, incubated and fixed by standard procedures described in detail elsewhere (Frixione *et al.* 1979; Frixione & Aréchiga, 1981). Briefly, following dissection the eyestalks were secured inside wide-mouthed vials (four to each vial) containing 6 ml of the test solutions and approximately 100 ml of a 95 % O_2 atmosphere. The test solutions containing serotonin and related compounds were made up in imidazole-buffered (pH 7·4) saline for freshwater crustaceans (Van Harreveld, 1936). Incubation was carried out for different intervals under regular fluorescent laboratory illumination, or in total darkness in a photographic room. The temperature was maintained at 18 °C throughout the incubation period. At the end of every interval the eyes were fixed by immersion in water at 85 °C. Although this traditional technique for fixation of crustacean eyestalks is unsuitable for fine histological studies, it still remains the method of choice for an instantaneous arrest of pigment position under any illumination condition, and subsequent measurement under low-magnification optics. The position of the pigment was inspected at 30× with a stereomicroscope in longitudinally bisected retinas, and quantitatively recorded as a proximal–pigment index (PPI) calculated from measurements taken with a micrometer as previously described (Frixione *et al.* 1979; Frixione & Aréchiga, 1981). The PPI may take values from 0·00, when the pigment is completely aggregated in the full dark-adapted state, to 1·00, when it is maximally dispersed in the light-adapted condition.

Preparation of photoreceptor membranes

The photoreceptor layers from 20 eyestalks were dissected out on a cold plate (0–4 °C) and homogenized in cold 30 mmol l^{-1} Tris–HCl buffer (pH 7·4) with a Teflon pestle glass homogenizer (0·15 mm clearance) operated at 700 rev. min^{-1}. The homogenate was centrifuged for 15 min at 600 *g* and the pellet (P-1), in which the bulk of pigment granules accumulated, was discarded. The supernatant (SN-1) was then centrifuged for 10 min at 12 000 *g*, and the second pellet (P-2) was either prepared for electron microscopy, as described below, or resuspended in the same buffer to a final protein concentration of 2 $\mu g\,\mu l^{-1}$, assayed by the method of Lowry *et al.* (1951). Na^+/K^+-ATPase activity was recovered predominantly in the P-2 fraction, whereas almost no measurable activity was detected in either the P-1 pellet or the final supernatant (SN-2).

Electron microscopy

The pellet obtained in the final centrifugation (P-2) was fixed for 2 h in cold 4 % glutaraldehyde made up in 0·1 mol l^{-1} sodium cacodylate (pH 7·0), released from the bottom of the tube, and split into small fragments with a razor blade. These pieces were rinsed in cold buffer and post-fixed for 30 min with 1 % osmium tetroxide in sodium cacodylate at 4 °C. The tissue was then washed in distilled water, dehydrated through a series of increasing concentrations of ethanol, transferred from pure ethanol to propylene oxide, and embedded in Araldite.

Silver thin sections were doubly stained with uranyl acetate and lead citrate, and micrographed with a Zeiss EM-9S2 electron microscope.

Na^+/K^+-ATPase activity determinations

50 μl samples of the P-2 fraction suspension described above were transferred to glass tubes containing 200 μl of the following reaction mixture (in mmol l^{-1}): Tris–HCl buffer (pH 7·4), 30; $MgCl_2$, 6; NaCl, 120; KCl, 20; ouabain, 0·1. A parallel series of samples did not receive ouabain in order to measure total ATPase activity. All samples were preincubated for 30 min at 37 °C in a Dubnoff shaker before the reaction was triggered by addition of 3 mmol l^{-1} ATP. The reaction was allowed to proceed for 10 min and then stopped with 20 μl of cold 50 % trichloroacetic acid. The samples were finally centrifuged for 5 min at 600 *g*, and inorganic phosphate in the supernatant was determined spectrophotometrically according to the method of Hosie (1965). All data shown here correspond to the ouabain-sensitive fraction of the total ATPase activity. The effect of serotonin was assessed in the presence of 10^{-4} mol l^{-1} pargyline, a monoamine oxidase inhibitor found to have no effect on Na^+/K^+-ATPase activity at this concentration in previous tests. All compounds tested were added to the reaction mixture during the 30 min preincubation period.

Results

Experiments with isolated eyes

Fig. 1 shows the observed positions of the proximal screening pigment in the crayfish retina when isolated eyes were exposed to light and darkness in the presence or absence of 10^{-3} mol l^{-1} serotonin. Incubation in either medium was started at time zero. The eyes were incubated for up to 90 min under continuous illumination, then transferred to darkness for up to 60 min, and finally exposed again to light for an additional hour. The eyes incubated in the presence of serotonin showed a partial aggregation of the pigment to the dark-adapted position after 15 min in the light, and this distribution was kept for as long as illumination was maintained in our experiments. The extent and rate of this movement correspond with those of the first phase of the normal migration to the dark-adapted position (Frixione *et al.* 1979). When illumination was interrupted, the pigment retreated to the extreme dark-adapted position. Eyes incubated in the presence of serotonin usually presented a more complete dark-adaptation than the controls, as judged both quantitatively by the values of the pigment index, and qualitatively by a 'cleaner' appearance of the region of the retina deserted by the pigment mass. With the onset of a further period of illumination, fully dark-adapted eyes showed a rapid pigment dispersion towards the light-adapted position. However, in the serotonin-treated eyes this movement did not proceed to completion as it did in the controls. Therefore, serotonin at this concentration exerted a dark-adapting influence over the pigment position for any of the illumination conditions tested. Lower concentrations of serotonin produced a les

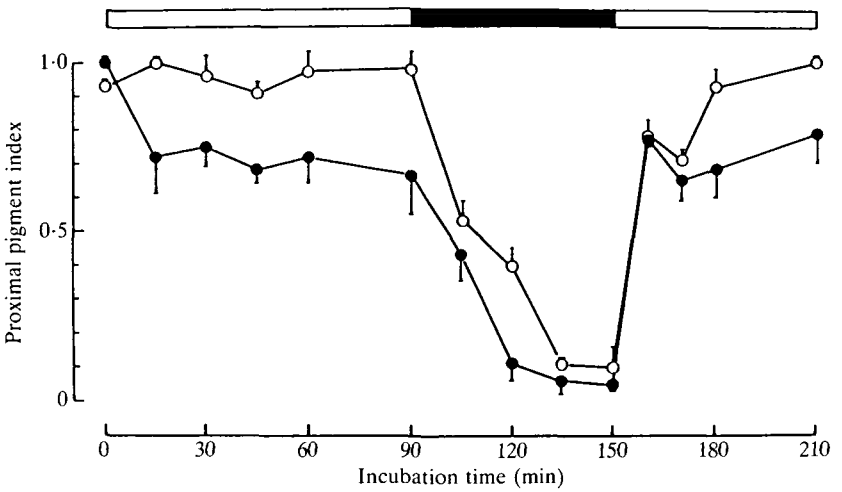

Fig. 1. Positions of the proximal screening pigment of crayfish eyes incubated with (●) or without (○) 10^{-3} mol l^{-1} serotonin under fluorescent laboratory light (white bar) and in darkness (black bar). Incubation in either medium started at time zero. Serotonin-treated eyes showed consistently lower values of proximal pigment index, revealing a dark-adapting influence of the amine under either illumination condition. Each point represents an average of at least four eyes, with standard deviation of the mean depicted by vertical lines.

consistent but still perceptible facilitation of pigment retraction (at 10^{-4} mol l^{-1}) or did not affect pigment behaviour (at 10^{-5} mol l^{-1} or lower).

Several classical agonists and antagonists of serotonin (see Hong *et al.* 1969; Grahame-Smith, 1971; Peroutka *et al.* 1981) were also tested for their effects on screening-pigment migration. The agonists quipazine and 5-methoxy-*N*,*N*-dimethyltryptamine (5-MDT) accelerated dark-adaptation during the second phase of the movement, although not with the same efficacy as serotonin (Fig. 2). Pigment dispersion elicited by illumination was not affected by 5-MDT, but a clear partial inhibition of this movement was observed with quipazine (Fig. 3). Statistical analysis of the data using Student's *t*-test revealed that the difference between quipazine-treated eyes and the controls after 30 min of illumination was significant at the $P < 0{\cdot}001$ level. The serotonin antagonists methysergide and cyproheptadine cancelled the dark-adapting influence of serotonin over pigment movements in either direction (Figs 4 and 5). Incubation of the eyes in saline containing the antagonists alone did not produce a noticeable effect.

Determinations of Na^+/K^+-ATPase activity on retinal membranes

Electron microscopy of the P-2 fraction used for ATPase determinations revealed a rather uniform field of vesiculated smooth membranes, which included onion-like multilamellar bodies but very few pigment granules and virtually no cytoskeletal elements (Fig. 6). These materials probably derive from all the cells contained in the photoreceptor layer of the compound eye, including tapetum cells

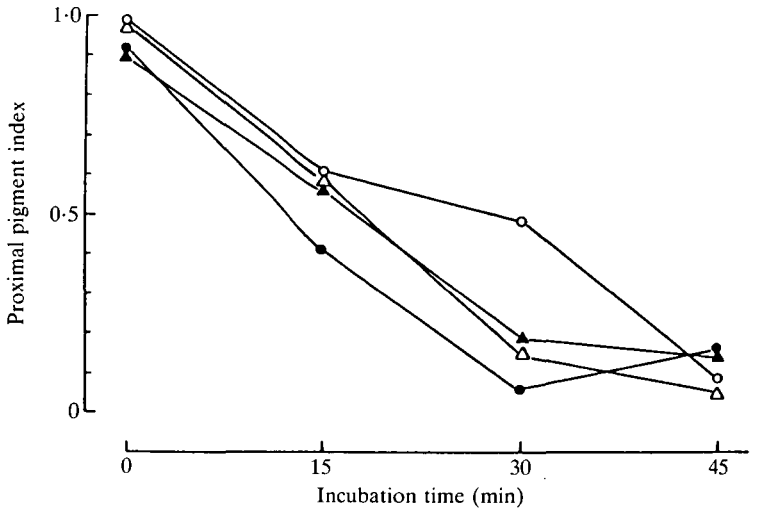

Fig. 2. Effect of serotonin and agonists upon pigment migration to the dark-adapted position in crayfish photoreceptors. Light-adapted eyes were incubated in the dark for the specified periods after time zero, in saline prepared with or without the compounds. Dark adaptation in the presence of serotonin (●), quipazine (▲) or 5-methoxy-*N*,*N*-dimethyltryptamine (△) was completed after only 30 min, when control preparations (○) were still midway through the normal course of migration. All drugs were at a final concentration of 10^{-3} mol l^{-1}. Each point represents an average of at least four eyes.

and haemocytes. Unfortunately, isolation of a relatively pure fraction from intact photoreceptors is not feasible at present because all attempts to dissociate the retinal tissue with trypsin, papain, low-Ca^{2+} medium and other treatments have so far been unsuccessful. Therefore, whole photoreceptor layers, carefully dissected free from adjoining structures, such as the main bodies of the crystalline cones and the first optic ganglion, were used as a source of membranes for Na^+/K^+-ATPase activity determinations. Although this technical limitation imposes some reservations with regard to the exact location of the enzyme, a simple inspection of the histology of the crayfish retina (see Krebs, 1972; Nässel, 1976) shows that the photoreceptors constitute most of the volume in this region of the compound eye. Accordingly, we believe that the observed activity resides predominantly, if not exclusively, in membranes from the photoreceptor cells.

The basal Na^+/K^+-ATPase activity (EC3.6.1.3) of the P-2 fraction, in the absence of serotonin or related compounds, was $1{\cdot}9 \pm 0{\cdot}19$ μmol Pi mg protein^{-1} h^{-1}. The effect of serotonin on the basal activity was tested over a concentration range of 10^{-9}–10^{-3} mol l^{-1} (Fig. 7). Activity was stimulated by 15% at 10^{-8} mol l^{-1} serotonin and increased almost sigmoidally with the logarithm of the amine concentration up to nearly 100% at 10^{-3} mol l^{-1}. A single but significant deviation occurred at 10^{-7} mol l^{-1}, which produced a stimulation of 45% over basal activity. The stimulation of Na^+/K^+-ATPase activity by serotonin was

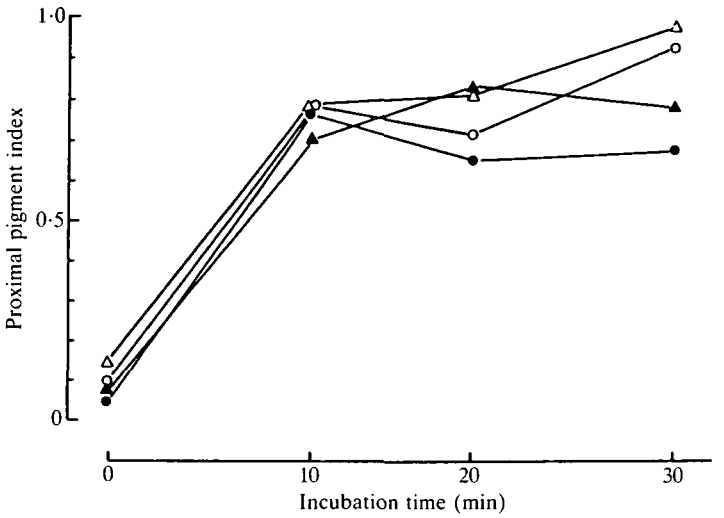

Fig. 3. Effect of serotonin and agonists upon pigment migration to the light-adapted position in crayfish photoreceptors. Dark-adapted eyes were exposed to light for the specified periods after time zero, in saline prepared with or without the compounds (all at 10^{-3} mol l^{-1}). Serotonin (●) and quipazine (▲) prevented the full pigment dispersion normally observed after 30 min of illumination in a control preparation (○); 5-MDT (△) did not affect this movement. Each point represents an average of at least four eyes.

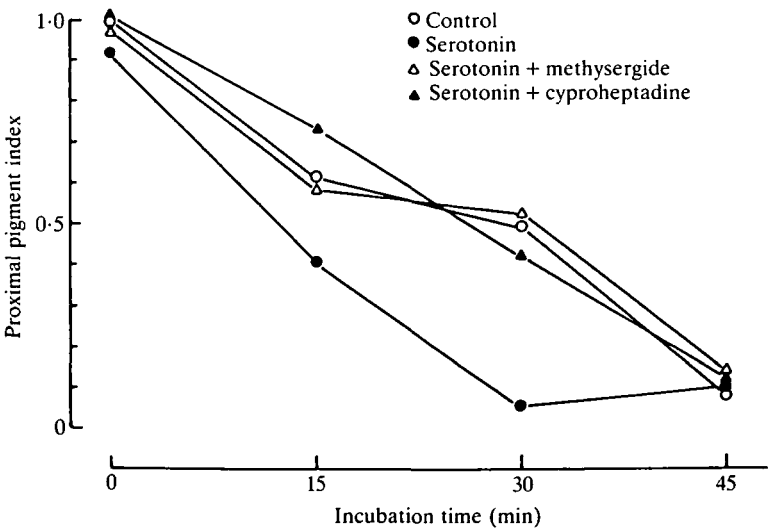

Fig. 4. Facilitation of pigment migration to the dark-adapted position by serotonin (●, 10^{-3} mol l^{-1}) is cancelled by the antagonists methysergide (△, 5×10^{-3} mol l^{-1}) and cyproheptadine (▲, 10^{-3} mol l^{-1}). Each point represents an average of at least four eyes.

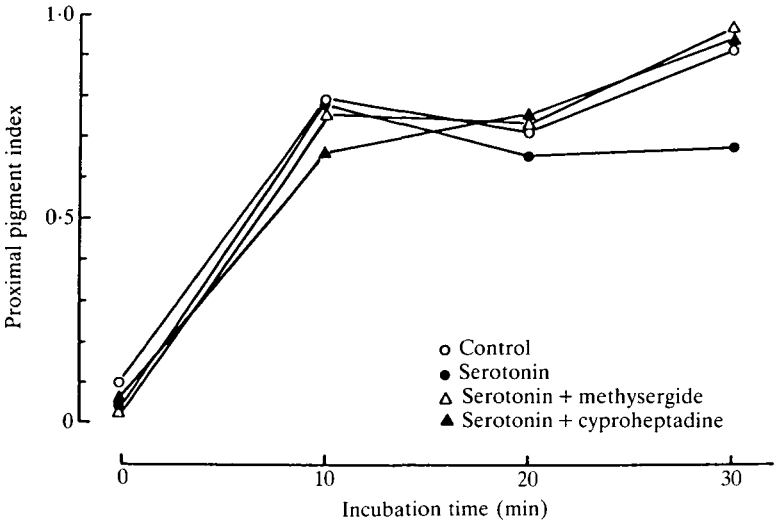

Fig. 5. Inhibition of pigment migration to the light-adapted position by serotonin (●, 10^{-3} mol l^{-1}) is cancelled by the antagonists methysergide (△, 5×10^{-3} mol l^{-1}) and cyproheptadine (▲, 10^{-3} mol l^{-1}). Each point represents an average of at least four eyes.

significant ($P<0{\cdot}05$ at 10^{-8} mol l^{-1} and $P<0{\cdot}01$ at the higher concentrations) compared with the controls (Student's *t*-test). Quipazine had a considerably stronger stimulatory effect than serotonin: 10^{-9} mol l^{-1} of the agonist enhanced the basal Na^+/K^+-ATPase activity by an amount equivalent to that to be expected for a serotonin concentration of 5×10^{-5} mol l^{-1} (Fig. 8). Methysergide, added simultaneously at concentrations from 10^{-9} to 10^{-5} mol l^{-1}, abolished the stimulatory effect of 10^{-3} mol l^{-1} serotonin or 10^{-7} mol l^{-1} quipazine.

Discussion

Serotonergic modulation of pigment position

The results described above show that serotonin induces a tendency for the screening pigment to retract to the dark-adapted position in crayfish photoreceptors. The PPI values were consistently lower in eyes exposed to serotonin or its agonists, either in the light or in darkness, and this effect was abolished by serotonin antagonists. These findings agree with the suggestion of a serotonergic modulation of pigment position in crustaceans (see Introduction). Therefore, a possible role of serotonin in the control of pigment position deserves careful consideration.

An interesting finding is that serotonin affected pigment position only at a concentration considerably higher than those effective on many other crustacean tissues *in vitro* (Beltz & Kravitz, 1986). The concentration of serotonin found clearly effective in our incubation bath is also greater than those that occur in

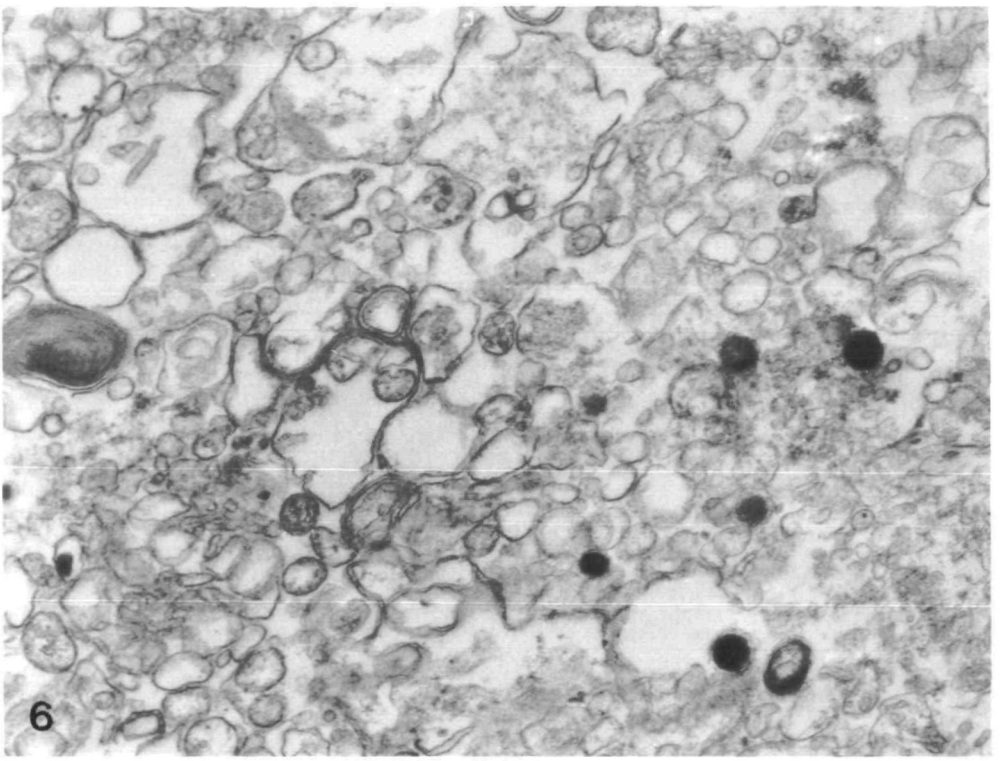

Fig. 6. Electron micrograph of the P-2 fraction of retinal homogenates used for ATPase determinations. The fraction appears to be composed of smooth-membraned vesicles of diverse sizes. Pigment granules were scarce in this material. ×32 000.

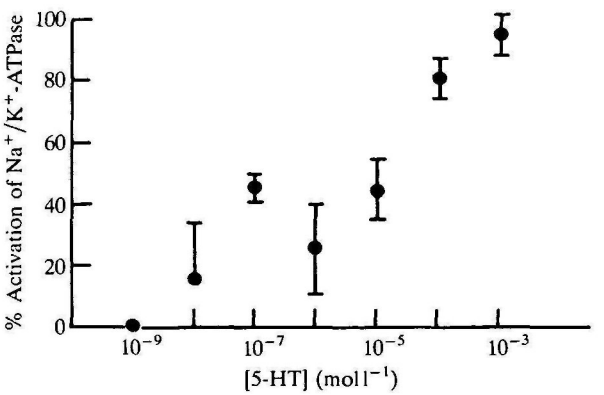

Fig. 7. Serotonin (5-HT) stimulation of Na^+/K^+-ATPase activity in the P-2 fraction of retinal homogenates. Each point represents an average of at least four determinations, with standard deviation of the mean depicted by vertical lines.

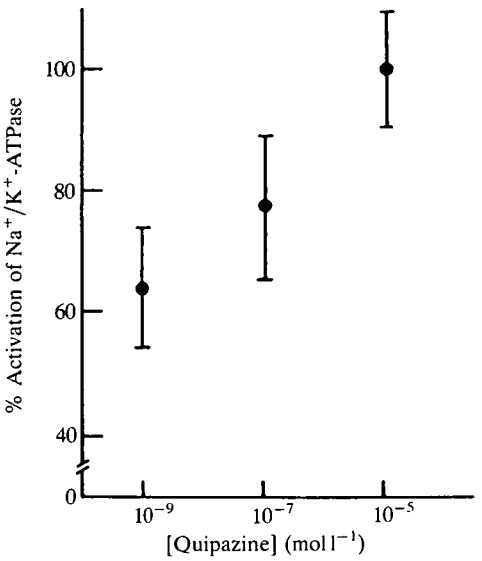

Fig. 8. Quipazine stimulation of Na^+/K^+-ATPase activity in the P-2 fraction of retinal homogenates. Each point represents an average of at least four determinations, with standard deviation of the mean depicted by vertical lines.

crayfish haemolymph (Elofsson *et al.* 1982). The relatively high levels of the amine required to produce an effect on the pigment could be explained by a limited diffusion of compounds from the bath to the photoreceptor layer inside the eyestalk, a limitation that would not occur with the eye *in situ*, where an efficient distribution of blood-borne metabolites is ensured by an intact vascular system. This argument is probably correct to some extent, because even small ions are subject to some restraint on inward and outward movements in perfused nervous tissues of crustaceans (Abbott *et al.* 1975). Also, drugs such as ouabain have to be used at high concentrations *in vitro* to affect crustacean photoreceptors (Stieve *et al.* 1977; Frixione & Aréchiga, 1981). Yet, the rapid effect of serotonin upon pigment position (Fig. 1) is an indication that diffusion barriers in the ocular tissue are not particularly restrictive for this amine. From our experience with this system, it is not unreasonable to assume a 5- to 10-fold difference in the concentration of serotonin between the bath and the interstitial fluid inside the retina. Since some effect was still observed with 10^{-4} mol l^{-1} serotonin, we believe the substance is able to produce a pigment response within a concentration range of 10^{-5}–10^{-4} mol l^{-1}. These levels of serotonin are comparable to those required to cause changes in the firing pattern of motoneurones of the lobster nerve cord (Harris-Warrick & Kravitz, 1984).

Since a relatively high concentration of serotonin is indeed necessary to influence pigment position, the levels circulating in the bloodstream are hardly sufficient to promote circadian migrations. However, concentrations much higher than these could probably build up near the plasma membrane of the photorecep

tors if the amine were acting as a locally delivered neuromodulator. The presence of efferent serotonergic fibres reaching the photoreceptors has recently been shown immunochemically in crayfish eyes (H. Aréchiga, E. Banuelos, E. Frixione, A. Picones & L. Rodríguez-Sosa, unpublished results). Similar neurones have also been found in the lamina of the blowfly optic lobe, where they are thought to exert a centrifugal influence over the photoreceptors in large portions of the visual field (Nässel *et al.* 1983). Part of this influence could be the activation of structural transformations involved in adaptation, including pigment migration, as suggested for octopamine in *Limulus* lateral eyes (Kass & Barlow, 1984). Evidence of serotonergic modulation exists also for the eye of *Aplysia* (Nadakavukaren *et al.* 1986) and for the retina of teleost fish (Dearry & Burnside, 1986).

Serotonergic modulation of Na^+/K^+-ATPase activity

Our experiments with the P-2 pellet of retinal homogenate demonstrate that serotonin is able to stimulate the Na^+/K^+-ATPase activity present in a cellular fraction composed almost entirely of membranes. Similar results at equivalent serotonin concentrations have been reported for synaptosomal preparations obtained from mammalian brain (Logan & O'Donovan, 1976; Lee & Phillis, 1977). Our findings add to a growing body of evidence for a modulatory action of serotonin and other neurotransmitters on the Na^+/K^+-ATPase of nerve cells (Yoshimura, 1973; Logan & O'Donovan, 1980; Rodríguez de Lores Arnaiz & Antonelli de Gómez de Lima, 1981, 1983; Hernández, 1979; Hernández & Chagoya, 1986). Although it is premature to discuss the possible effect of serotonin at the molecular level in this system, it is nevertheless pertinent to point out that the high sensitivity to quipazine, as well as the abolition of the serotonergic effect on enzyme activity by methysergide, suggest an effect mediated by a specific receptor – perhaps of the 5-HT_1 type – as proposed in view of a similar pharmacological profile for synaptosomal membranes from rat brain (Hernández, 1980, 1982, 1987).

Involvement of serotonin and Na^+/K^+-ATPase activity in the control of screening-pigment position

It is now well established that the vectorial transport of intracellular components is keenly dependent upon the ionic balance in the cytoplasm. In particular, the internal levels of Ca^{2+} seem to play a prominent role in the regulation of pigment granule movements in arthropod photoreceptors (Kirschfeld & Vogt, 1980; Frixione & Aréchiga, 1981) and chromatophores (Luby-Phelps & Porter, 1982), as well as in axoplasmic transport (Ochs, 1982). In turn, the regulation of cytoplasmic Ca^{2+} concentration appears to be sensitive to the Na^+ levels inside some of these cells. Evidence for a Na^+-dependent modulation of internal Ca^{2+} levels exists in the case of invertebrate photoreceptors (Waloga *et al.* 1975; Bader *et al.* 1976). A Na^+-elicited Ca^{2+} release from intracellular stores has been proposed as a likely mechanism for the coupling of light adaptation with pigment dispersion in crayfish photoreceptors (Frixione & Aréchiga, 1981) and for the Na^+-sensitivity of

axoplasmic transport in batrachotoxin-treated nerves (Worth & Ochs, 1982). The main Ca^{2+} stores have been identified as smooth endoplasmic reticulum in several photoreceptors (Perrelet & Bader, 1978; Walz, 1979, 1982*b*; Ungar *et al.* 1984; Coles & Rick, 1985; Somlyo & Walz, 1985) and in other nerve cells (Duce & Keen, 1978; Chan *et al.* 1984; Burton & Laveri, 1985). An inhibitory influence of Na^+ over the Ca^{2+} storing ability of the smooth endoplasmic reticulum has been shown in photoreceptors of leech (Walz, 1982*a*) and crayfish (Frixione & Ruiz, 1988). It is thus conceivable that any stimulus producing fluctuations in the levels of internal Na^+ could affect pigment position in arthropod photoreceptors through modulations of internal Ca^{2+} level.

Within this conceptual framework, enhancement of Na^+/K^+-ATPase activity – and thus Na^+ extrusion through the plasma membrane – by serotonin would be expected to decrease the levels of Na^+, and therefore of Ca^{2+}, in the cytoplasm of crayfish photoreceptors, with a consequent facilitation of pigment aggregation to the dark-adapted position. Nocturnal activation of a serotonergic efferent input to the photoreceptors, probably driven by the same circadian oscillator that increases serotonin levels in the eyestalks of some crustaceans after dusk (Fingerman & Fingerman, 1977), would favour a more complete dark-adapted position of the pigment at night, even under constant darkness, as discovered by Bennitt (1932). Demonstration of a circadian efferent input mediated by serotonin over the photoreceptors of crayfish is obviously necessary for any further exploration of this hypothesis.

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