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Opsin-like immunoreactivity in the circadian pacemaker neurons and photoreceptors of the eye of the opisthobranch mollusc *Bulla gouldiana*

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Abstract. Circadian pacemaker cells in the eyes of the opisthobranch mollusc *Bulla gouldiana* generate a near 24-h rhythm in the frequency of optic nerve impulses. Previous electrophysiological studies suggest that these basal retinal neurons are intrinsically photosensitive and transduce light signals that shift the phase of their pacemaker mechanism. To test whether the pacemaker neurons contain opsin-like proteins, several polyclonal antibodies that recognize opsins of vertebrate photoreceptors have been tested on histological sections of the eye and on the neurons in primary cell culture. The antibodies label both the pacemaker cells and the large distal photoreceptors that surround the lens. Immunoblot analyses of the proteins of the eye have identified a single band at 62±4 kDa. These opsin antibodies may label the photopigment used in the entrainment of the circadian pacemaker.

Key words: Opsin – Circadian pacemaker – Polyclonal antibodies – Retina – *Bulla gouldiana* (Mollusca)

Introduction

The eyes of several opisthobranch molluscs contain a pacemaker that generates a circadian rhythm in the frequency of spontaneous optic nerve impulses (Jacklet 1969; Eskin and Harcombe 1977; Eskin 1979; Block and Roberts 1981; Block and Wallace 1982). These impulses are large (~100 μV) compound action potentials that result from the simultaneous firing of a population of electrically coupled retinal cells. In the opisthobranch mollusc *Bulla gouldiana*, the compound action potentials and the rhythm of their frequency are both generated by the basal retinal neurons, a population of approximately 100 circadian pacemaker cells near the origin of the optic nerve (Block et al. 1984; Michel et al. 1993). The basal retinal neurons generate compound action potentials spontaneously or by depolarizing in response to light (Block and Wallace 1982) and they appear to regulate circadian rhythms in the frequency of other retinal impulses (Geusz and Page 1990; Geusz and Block 1992).

Like all circadian pacemakers, the oscillator in the *B. gouldiana* eye entrains to cycles of light and dark, including the 24-h light/dark cycle of the environment. The cells and photopigments used in capturing the entraining light signal, the first step of the entrainment process, have been examined in diverse animal groups, such as molluscs (Waser 1968; Jacklet 1980; Beiswanger et al. 1981), insects (cockroaches: Mote and Black 1981; moths: Bruce and Minis 1969; flies: Frank and Zimmerman 1969, Zimmerman and Goldsmith 1971, Klemm and Ninnemann 1976), reptiles (Foster et al. 1993), birds (Deguchi 1981; Foster and Follett 1985; Okano et al. 1994; Max et al. 1995) and mammals (bats: Joshi and Chandrashekaran 1985; hamsters: Takahashi et al. 1984; mice: Foster et al. 1991). Nevertheless, no photopigment used in the entrainment of animal circadian rhythms is known with certainty.

In *B. gouldiana*, phase shifts to light exposure seem to result from photon capture within the basal retinal neurons (Block et al. 1984). Eyes maintained in artificial sea water containing elevated levels of Mg^{2+} and reduced levels of Ca^{2+} continue to generate compound action potentials in response to light, suggesting that the basal retinal neurons do not respond to light by way of chemical synaptic transmission from another cell type (Block and Wallace 1982). Similarly, the basal retinal neurons remain photosensitive in eye preparations with the approximately 1000 microvillous photoreceptors of the distal portion of the eye cut away and the circadian rhythm in these surgically-reduced eyes can still be phase-shifted by light (Block and McMahon 1984). Finally, compound action potentials are more readily elicited by microillumination of the base of the eye than of the photoreceptor layer (Block and McMahon 1983).

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Spectral sensitivity measurements suggest that the photopigment involved in both the photoresponse of the basal retinal neurons and the entrainment of the pacemaker is based on a form of the protein opsin (Geusz and Page 1991), which binds the chromophore 11-cis retinaldehyde (Wald 1968). With no knowledge of the exact nature of the chromophore, i.e. whether it is derived from vitamin A1 or A2, it is not yet known whether this photopigment is a rhodopsin or a porphyropsin. The action spectra of the *B. gouldiana* eye suggest that the same type of photopigment is found in the photoreceptors and at the base of the eye (Geusz and Page 1991). In the present study, we have tested whether the basal retinal neurons are recognized by well-characterized polyclonal antibodies raised against the opsins of bovine rod, chicken cone or chicken rod photoreceptor cells.

Materials and methods

Animals and eye removal

Bulla gouldiana were obtained from Marinus (Long Beach, Calif., USA) and maintained at 15°C in artificial sea water (Instant Ocean). Animals were maintained under cycles of 12 h fluorescent light and 12 h darkness in order to entrain the circadian system. Animals were relaxed by injection with isotonic $MgCl_2$ and the eyes were removed and placed in artificial sea water consisting of 395 mM NaCl, 10 mM KCl, 10 mM $CaCl_2$, 50 mM $MgCl_2$, 28 mM Na_2SO_4, 30 mM HEPES (N-[2-hydroxyethyl]piperazine-N′-2-ethanesulphonic acid), 100 000 U/l penicillin, and 100 mg/l streptomycin, adjusted to pH 7.8, and filtered at 0.2 μm.

Antibody production

Four polyclonal antisera were used in this study: CERN-874 and CERN-906 both generated against chicken cone opsins and CERN-858 and CERN-901 generated against bovine rod opsin and chicken rod opsin, respectively. The method used to produce and test the antibodies has been described in detail previously (Foster et al. 1993). CERN-901 was purified as described for the cone opsins (Foster et al. 1993). Briefly, cone or rod photopigments were purified on a sucrose gradient, extracted with 20 mM dodecylmaltoside, purified over ConA-Sepharose, eluted and then injected into rabbits under dim red light. After 24 h in darkness following the injection, the animals were returned to a standard cycle of 12 h light and 12 h dark. Antisera were collected and tested for specificity by immunoblot and immunohistochemical analysis. The specificity was high enough to differentiate cone photoreceptors from rod cells by their relative affinities for the antibodies. Preadsorbed antibody used for immunocytochemistry was prepared by mixing 5.5 ml CERN-874 (1:4000) with 0.3 mg chicken cone protein, which has been shown to block immunolabelling (Foster et al. 1993).

Immunolocalization on paraffin sections

After removal of the lens through an incision in the eye sheath, eyes were fixed overnight at 4°C in 2% paraformaldehyde in phosphate-buffered saline (PBS) with the osmolarity adjusted to 1020 mmol/kg with sucrose, dehydrated in an ethanol series, cleared in methyl benzoate and embedded in Paraplast (Monoject Scientific, St. Louis, Mo., USA). Sections (10 μm in thickness) from each eye were placed alternately on two slides previously coated with alum-gelatin. Paraplast was removed with xylene (30 min) and the sections were rehydrated through an ethanol series, rinsed with 0.1% Triton X-100 (Sigma, St. Louis, Mo., USA) in PBS (PBS-T), placed in 0.3% H_2O_2 in PBS (30 min at 4°C), rinsed with PBS-T and then incubated in normal goat serum (1:30, 30 min at room temperature). One of these slides was transferred directly to primary antibody, whereas the other was left in normal goat serum. The sections were incubated for 20 h at 4°C in a humid chamber. After being rinsed with PBS-T, the sections were placed in biotinylated secondary antibody for 1 h at 4°C (goat anti-rabbit, Vectastain Elite kit; Vector Laboratories, Burlingame, Calif., USA), rinsed again with PBS-T and placed in the avidin-biotin complex reagent (Vectastain; Vector Laboratories) for 1 h at 4°C. The sections were rinsed with TRIS buffer (Sigma) and then placed in a solution of 25 mg diaminobenzidine (DAB) in 100 ml TRIS buffer with 20 μl H_2O_2 for 90 s. The slides holding the experimental and control sections for a given eye were moved through the antibodies and reagents together in a slide holder to ensure identical treatment. After being rinsed with TRIS buffer, the sections were dehydrated and mounted for viewing. In some cases, both control and experimental sections were treated with osmium after the diaminobenzidine reaction to intensify the label.

Immunolocalization in cell cultures of basal retinal neurons

Cell cultures of basal retinal neurons were similar to those described previously (Michel et al. 1993). The eyes were surgically reduced in artificial sea water with iridectomy scissors by removing the lens, distal photoreceptors and entire pigment-granule layer. The base of the retina, containing the basal retinal neurons and the neuropil, was then freed from the optic nerve and eye sheath, treated for 1 h in 0.01% protease (Sigma) in artificial sea water at room temperature and transferred to cell culture medium on a glass coverslip coated with poly-D-lysine. The culture medium consisted of L-15 with salts corrected for use with marine molluscs and was similar to that of Schacher and Proshansky (1983), except that haemolymph was not used and 0.1 mg/ml gentamicin and 30 mM HEPES were added. The pH was adjusted to 7.8. The basal retinal tissue was triturated gently in the medium with fire-polished glass pipettes to disperse the cells. The cultures were maintained under constant light at room temperature for 18–48 h before proceeding with antibody labelling.

The cell cultures were fixed for 15 min at room temperature in 4% paraformaldehyde in PBS with 30% sucrose. The cells were then rinsed in artificial sea water, permeabilized with 0.25% Triton X-100 in PBS for 5 min, rinsed in PBS, incubated for 1 h in normal goat serum in PBS and then transferred either directly to primary antiserum for 1 h or, in the case of control cultures, to normal goat serum. The coverslips were inverted and placed over 100-μl drops of antiserum or normal goat serum on Parafilm and kept in a sealed chamber. Next, the cells were rinsed in PBS three times and moved to biotinylated goat anti-rabbit antisera for 1 h, rinsed in PBS and then placed in the avidin-biotin complex for 1 h. All of these initial steps were at room temperature. The cells were then rinsed in cold (4°C) TRIS buffer, reacted with cold DAB solution for 90 s, rinsed, dehydrated, transferred to xylene for about 1 min and then mounted on glass slides.

Additional cell cultures of basal retinal neurons were treated in the same manner as the DAB-stained cells but were instead moved to avidin-fluorescein isothiocyanate (avidin-FITC, 1:100; Vector Laboratories) for 1 h after treatment with the secondary antibody. These cells were rinsed, mounted on glass slides with Vectashield (Vector Laboratories) and then viewed with a Sarastro 2000 confocal laser scanning microscope (Molecular Dynamics, Sunnyvale, Calif., USA).

Western blots

For Western analysis, 20 eyes in artificial sea water were frozen in liquid nitrogen after first removing the lens. A second group of 20

eyes was also prepared and used in a replicate experiment. An additional ten eyes were surgically reduced by removing all the distal photoreceptors and the entire pigment-granule layer before freezing.

For protein extraction, 0.5 ml PIPES buffer (5 mM piperazine-N,N′-bis[2-ethanesulphonic acid], 40 mM NaCl, 0.5 mM $CaCl_2$, 10 mM ethylenediamine tetraacetic acid, and 10 µM leupeptin, pH 6.5) was added to 10 eyes. The suspension was homogenized in an Eppendorf tube with a small pair of scissors and rotated slowly for 1 h at 4°C in a nitrogen atmosphere. After subsequent centrifugation (10 000 *g*, 20 min, 4°C), the supernatant containing most of the soluble proteins was carefully discarded. A 50-µl aliquot of extraction buffer (5 mM dithioerythritol and 1% sodium dodecylsulphate in PIPES buffer, pH 6.5) was added to the pellet. After a 1-h incubation at room temperature with slow rotation under nitrogen, the mixture was centrifuged (10 000 *g*, 20 min, 4°C) and the supernatant (membrane-protein extract) was removed and directly applied to a sodium dodecylsulphate-polyacrylamide gel or stored under nitrogen at –20°C.

For sodium dodecylsulphate-polyacrylamide gel electrophoresis, 20 µl membrane-protein extract was mixed with 10 µl polyacrylamide gel electrophoresis buffer (135 µl 0.5 M TRIS-HCl, pH 6.8, 480 µl 87% glycerol, 400 µl 0.06% [w/v] bromophenol blue). Aliquots of the resulting solution (maximally 10 µl) were applied to a 12% homogeneous sodium dodecylsulphate-polyacrylamide mini-gel (Mini Protean II; BioRad, Melville, N.Y., USA) and run for 1–2 h at 200 V.

For immunodetection, the proteins separated in the polyacrylamide gel were blotted onto nitrocellulose (1 h, 100 V) by means of standard techniques (Burnette 1981). Nonspecific protein-binding sites were saturated by incubating the blot with 5% bovine serum albumin in PBS for 2 h at room temperature. Blots were incubated overnight at room temperature with the primary antibody diluted 1:500 in PBS. After being washed in PBS (3×10 min), the blots were incubated with the secondary antibody (swine anti-rabbit immunoglobulins, 1:300; DAKO, Glostrup, Denmark). Blots were washed again (3×10 min) and reacted with rabbit horseradish peroxidase antibodies (1:500; DAKO). Blots were washed once more (3×10 min) and the bound horseradish peroxidase complex was visualized with 4-chloro-1-naphthol (15 mg 4-chloro-1-naphthol in 5 ml cold methanol mixed just before use with 25 ml PBS containing 15 µl 30% H_2O_2) by incubation for 10–20 min at room temperature. Finally, the blots were washed (3×10 min) in double-distilled water and dried between filter papers. Chemiluminescent immunoblots were analysed with a BioRad GS-670 imaging densitometer.

Results

Immunocytochemistry

Antibody CERN-874 was the most effective of the four antibodies at labelling the basal retinal neurons and the photoreceptors in paraffin sections (Fig. 1), giving positive results, at a dilution of 1:4000, in sections from 5 out of 5 eyes. The labelling with CERN-874 was found primarily in cytoplasmic structures of the basal retinal neurons and along the microvillous membranes of the photoreceptors. Some labelling was also found in the cytoplasm of photoreceptors distal to the nucleus and in the eye capsule or sheath. Glial-like cells surrounding the basal retinal neurons were not labelled. Preadsorption of the antibody with excess antigen reduced labelling of the basal retinal neurons and photoreceptors to background levels (Fig. 2).

Antibody CERN-901 also labelled the basal retinal neurons and photoreceptors in paraffin sections. Label was observed in 2 out of 4 eyes at a dilution of 1:2000; labelling was not observed at a dilution of 1:8000. Both antibodies CERN-858 (1:2000, *n*=1]) and CERN-906 (1:2000, *n*=4; 1:8000, *n*=1) failed to clearly label the basal retinal neurons or the photoreceptors in paraffin sections.

All four antibodies labelled the basal retinal neurons in dispersed cell cultures (Figs. 3, 4). When DAB was used with the avidin-biotin-complex method on fixed

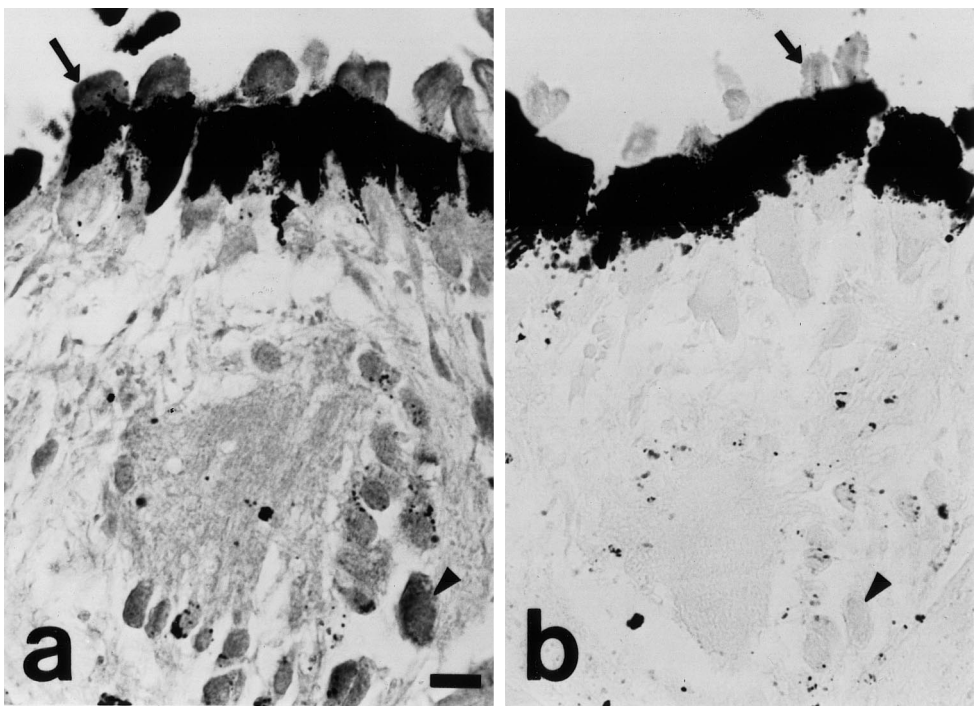

Fig. 1a, b. Sections of paraffin-embedded eyes labelled with CERN-874. **a** Some basal retinal neurons (*arrowhead*) and outer regions of distal photoreceptors (*arrow*) are labelled. **b** Control section from the same eye with primary antibody omitted. The microvillous outer region of distal photoreceptors (*arrow*) receives light through a spherical lens that was removed prior to fixation. The dark pigment-granule layer separates the outer region of distal photoreceptors from the basal retinal neurons (*arrowhead*), which are interspersed with smaller glial-like cells at the base of the eye. Osmium intensification. ×400. *Bar:* 20 µm

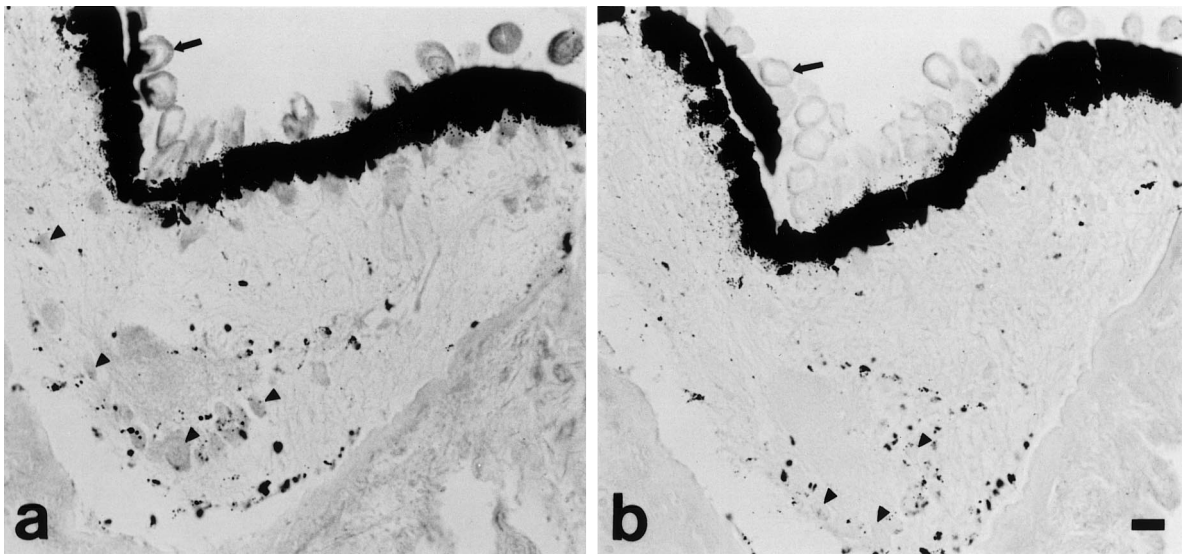

Fig. 2a, b. Preadsorption of CERN-874 with antigen eliminates positive labelling of the basal retinal neurons (*arrowheads*) and the distal photoreceptors (*arrows*). **a** CERN-874. **b** Preadsorbed CERN-874. Osmium intensification was not used. ×200. *Bar:* 20 μm

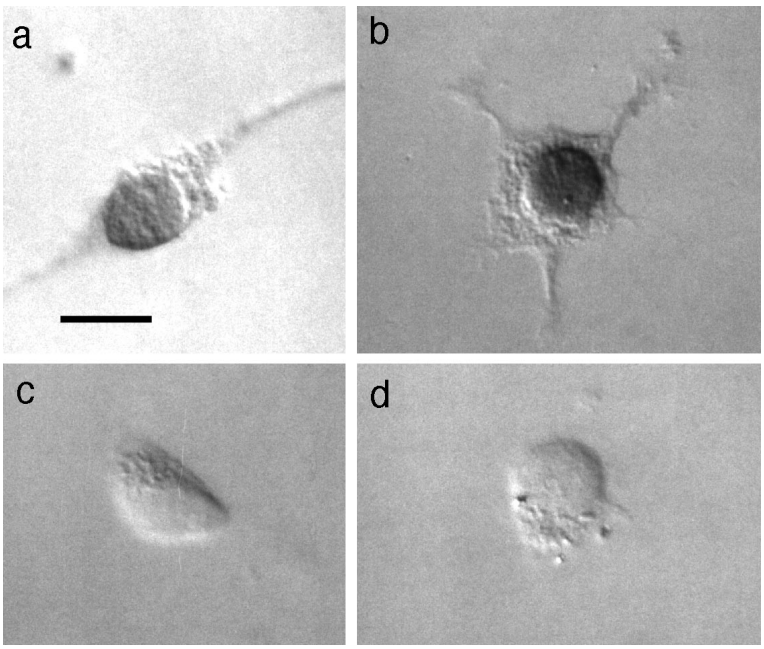

Fig. 3a–d. Immunolabelling of whole basal retinal neurons in primary cell culture. Cells labelled with CERN-858 at dilutions of 1:500 (**a**) and 1:1000 (**b**). Strongest labelling was near and around the nucleus. Control cultures with primary antibody omitted (**c, d**). Differential interference-contrast was used to emphasize cell structures. Cells have not yet regrown their processes. ×460. *Bar:* 25 μm

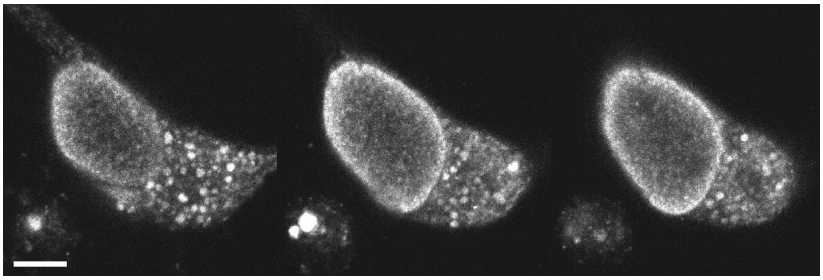

Fig. 4. Confocal sections through a basal retinal neuron immunolabelled with CERN-901 at a dilution of 1:1000. The perimeter of the nucleus and small cytoplasmic structures are labelled. The interval between sections is 1 μm. ×1000. *Bar:* 5 μm

and permeabilized cells, the label was highest near the nucleus and in small cytoplasmic structures (Fig. 3), whereas the plasma membrane was not labelled. The effectiveness of the antibodies was judged as CERN-901 > CERN-874=CERN-858=CERN-906 (CERN-901 at 1:500, *n*=1; 1:1000, *n*=1; CERN-874 at 1:500, *n*=2; 1:1000, *n*=1; CERN 858 at 1:500, *n*=5; 1:1000, *n*=2; CERN-906 at 1:500, *n*=2). No labelling was observed when normal goat serum was used in place of the primary antibodies. Although the cultures of basal retinal neurons were fixed at various times during the circadian cycle, there was no obvious difference in labelling between cultures.

To determine whether the antibodies were localized at the edge or the interior of the nucleus, basal retinal neurons were grown in culture and then examined using confocal microscopy and immunocytochemistry with FITC as the fluorophore. Image sections made through the basal retinal neurons revealed clear labelling of cytoplasmic structures, together with an area at the edge of the nucleus (Fig. 4). The cells appeared to be better labelled with CERN-901 than with CERN-906. Fluorescence from basal retinal neurons was barely detectable when normal goat serum was substituted for primary antibody; images were made with the same laser and photomultiplier-tube settings as those used for the antibody-treated cells. The cell-culture conditions were not optimal for the survival of the distal photoreceptors, so these cells were not tested with the antibodies.

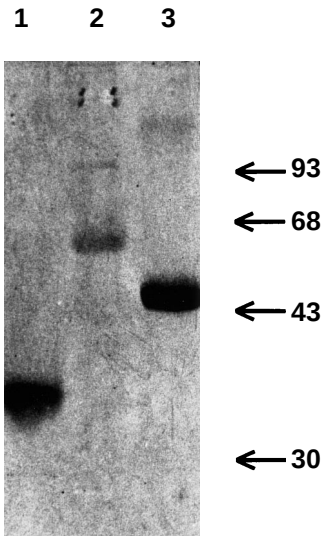

Fig. 5. Immunoblot of eye proteins using CERN-901. *Lane 1* Bovine rod outer segment membranes (control), *lane 2* protein extracted from three *B. gouldiana* eyes, *lane 3* squid rhabdomeric membranes (control). Positions of calibration proteins are indicated. A semilog plot of migration distance versus molecular weight (not shown) yields the expected molecular weights of 39±2 kDa for bovine rhodopsin and 49±3 kDa for squid rhodopsin. The same analysis yields 62±4 kDa (*n*=4) for the immunopositive band in *B. gouldiana* eye. This band was immunopositive with all four antisera, with labelling intensity decreasing in the order CERN-901 > CERN-906 > CERN-874 > CERN-858. Bovine and squid rhodopsins only react with CERN-901 and CERN-858

Immunoblotting

The antibodies were tested on Western blots of the eye to determine the specificity of the labelling and to help identify the protein involved. All four antibodies labelled very specifically a band corresponding to a 62±4 kDa protein (Fig. 5). This result was replicated with a second group of eyes and the same band was detected with CERN-901. A faint band in the 90 to 110-kDa range was present with all antisera; this could represent a dimer which, extrapolating from vertebrate pigments, should migrate with an apparent molecular weight in the range of 100–120 kDa.

Preadsorption of the CERN-858 antibody with antigen greatly reduced labelling of the 62-kDa band of *B. gouldiana* and the bovine rhodopsin band (Fig. 6). There were no detectable proteins when pre-immune serum was used in place of CERN-901 in an additional control immunoblot (not shown).

Faint labelling was observed in preparations made from surgically reduced eyes in which the distal portion of the eye, including the microvillous photoreceptors and the pigment-granule layer, had been removed. This extract of up to 5 surgically reduced eyes did not give a reliable Western blot reaction.

The immunohistochemical data suggest that the amount of photopigment in the basal retinal neurons is less than 5% of that in the photoreceptors. Each eye contains at least 1000 photoreceptors and about 100 basal retinal neurons (Block and Wallace 1982) and the diam-

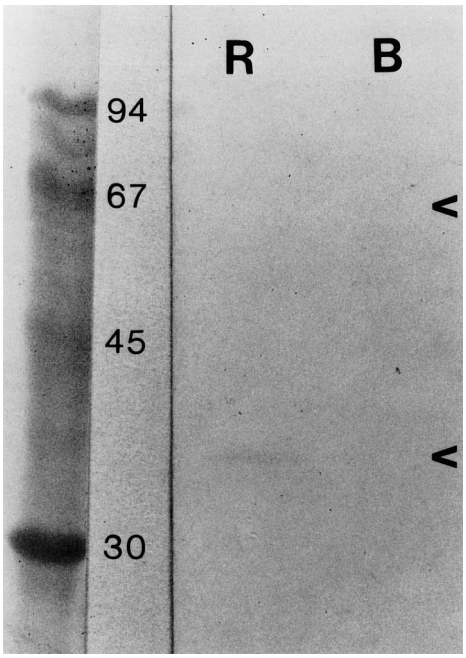

Fig. 6. Preadsorption of CERN-858 with antigen strongly reduces detection of proteins from *B. gouldiana* eye extract (*B*) and bovine photoreceptor membranes (*R*) in this immunoblot. *Arrowheads* mark the positions of the bands for *B. gouldiana* and bovine proteins identified in Fig. 5

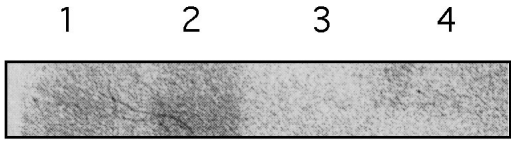

Fig. 7. An immunoreactive band in protein extracts from surgically reduced *B. gouldiana* eyes; chemiluminescence-detection method. *Lanes 1, 2* Protein from 5 reduced eyes and 2 intact eyes, respectively, *lane 3* empty, *lane 4* extract from bovine rod outer segments, showing the bovine rhodopsin dimer (68±4 kDa). High background interferred with conclusive determination of the bands in *lanes 1, 2*

eter of each distal photoreceptor outer region alone (30 μm) is larger than most basal retinal neurons. Furthermore, the microvilli of the distal photoreceptors, which basal retinal neurons lack, contain a considerable area of photosensitive membrane. We estimate that at least 20 reduced eyes would be required to obtain detectable bands, although such extracts would greatly overload the gel. By using a more sensitive (about 25-fold) chemiluminescence-detection method, we have been able to distinguish an immunopositive band in extracts of 5 reduced eyes at the correct position but, under these conditions, the high background does not allow unambiguous identification (Fig. 7).

Discussion

Site of photon capture

The labelling of cytoplasmic structures of the basal retinal neurons with cone opsin antibodies reaffirms that the circadian pacemaker neurons contain a photopigment based on opsin. The results agree with prior studies relying on electrophysiological measurements of the light response of the basal retinal neurons; these studies have suggested that this cell type is intrinsically photosensitive (Block and Wallace 1982; Block et al. 1984; Geusz and Page 1991). Labelling of the basal retinal neurons is greatest within cytoplasmic structures that might be light-sensitive organelles.

Although basal retinal neurons lack an obvious photoreceptive structure, such as the microvillous membranes of typical invertebrate photoreceptors, light may be captured in these cells by the membranous organelles described in ultrastructural studies of the eye (Jacklet and Colquhoun 1983; Bogart 1992; S. B. S. Khalsa, unpublished observations). These structures resemble the lipochondria that are found in the neurons of the abdominal ganglia of *Aplysia californica* and that undergo morphological changes in response to light (Brown et al. 1975; Henkhart 1975); these lipochondria are also labelled by antibodies generated against squid opsin (Robles et al. 1986). Preliminary studies with this squid opsin antibody have also shown labelling in the *B. gouldiana* eye (Robles et al. 1988).

If the basal retinal neurons can be shown to contain lipochondria that respond to light, then these structures probably capture photons used in the entrainment of the circadian pacemaker. Further analysis of these putative lipochondria awaits immunolabelling at the ultrastructural level, which we predict will stain the aforementioned membranous organelles.

Antibody CERN-874 labels both the basal retinal neurons and the membranes of the microvilli in the distal photoreceptors. Small structures in the distal portion of the photoreceptor cytoplasm are also labelled with CERN-874. These structures are probably the photic vesicles used to transport photopigment to the membrane of the microvilli (Eakin and Brandenburger 1978; Schwemer 1986). Such a function for these labelled structures would account for their presence on the side of the photoreceptor soma oriented towards the outer regions. We have found no evidence of opsin in small cells of the retina (less than 10 μm in diameter), some of which may be the H-type cells described previously (Block et al. 1984; Geusz and Block 1992).

Labelling of the eye capsule, which is continuous with the nerve sheath, could be attributable to photoreceptive cells in the sheath, although none has yet been characterized. A transient autofluorescence has been observed in the distal photoreceptors and the eye sheath of *B. gouldiana* (Geusz 1989) after treatment that induces fluorescence from N-retinyl proteins derived from rhodopsin (Eakin and Brandenburger 1978). Moreover, spontaneous impulses that are inhibited by light have been recorded from sections of the optic nerve that still contain the nerve sheath but no retinal cells (Geusz and Page 1990), again suggesting that photoreception could occur in this outer tissue layer. It is not known whether any phototransduction in the eye sheath is involved in the entrainment of the circadian pacemaker neurons.

Opsins of B. gouldiana

Previous studies have shown that light responses from both the microvillous photoreceptors and the basal retinal neurons fit the expected absorption spectrum for an opsin-like photopigment absorbing maximally near 490 nm (Geusz and Page 1991). Microspectrophotometry has also been used to identify an opsin-like absorption spectrum in the distal photoreceptors, a spectrum that has a maximum at 493 nm (Bogart 1992). The similar labelling of the distal photoreceptors and the basal retinal neurons with the antibodies suggests that the same or similar opsin-like protein is present in both cell types.

The lack of immunocytochemical labelling with antibodies CERN-906 and CERN-858 in paraffin sections could have been a result of masking of their corresponding epitopes by cellular components or of loss of the epitopes during histological treatments. In contrast, the *B. gouldiana* proteins are probably in a more native state when immunolabelling is performed on cells maintained in culture, since these cells are only fixed and permeabilized. Both of these antibodies have been used in limited studies with paraffin sections and may require a different protocol.

A single protein band is labelled in our Western blot analysis and has a molecular weight (62±4 kDa) that is within the range observed for opsin molecules of molluscs (Stavenga and Schwemer 1984); these molecules generally are of a higher molecular weight than those of vertebrates. The very low amount of photopigment present in the basal retinal neurons impedes unambiguous identification by Western blot analysis but all available evidence suggests that it is the same protein as that in the distal photoreceptors. A molecular weight of 62 kDa would, however, put this pigment at the highest end of the molecular weight range of photoreceptor pigments and makes it an interesting candidate for further studies directed towards primary structure and transduction mechanisms. Possible glycosidation of the protein has not been examined and identification of the amino-acid sequence will also be necessary before confirmation of this protein as an opsin.

The apparent ability of CERN-874, CERN-901, CERN-858 and CERN-906 to identify both vertebrate and invertebrate photopigments distinguishes them from many of the antibodies that have been generated against vertebrate opsins. It is possible that the antibodies recognize sites either common among invertebrate and rod opsins or between invertebrate and cone opsins, making them valuable in functional studies of the structure of invertebrate opsins. The epitopes that they recognize in invertebrates may have evolved independently of those in vertebrates or they may represent structures that are still retained in the vertebrate opsins. The antibodies need not, however, identify structures common to both rod opsins and cone opsins. Instead, different rod and cone epitopes may be reactive to these polyclonal antisera.

Light transduction and pacemaker entrainment

The immunological characterization of the photopigment used in photon capture by the basal retinal neurons suggests that a typical invertebrate transduction mechanism is used in the entrainment pathway of the circadian pacemaker of *B. gouldiana*. Identification of the photopigment used in entrainment is difficult in many organisms, because circadian pacemakers typically rely on extra-retinal or non-visual photoreceptors that are not well characterized (reviewed by Ninnemann 1979; Page 1982; Foster and Menaker 1993).

Earlier attempts to detect light responses from dispersed basal retinal neurons have been unsuccessful (Michel et al. 1993). Recently, however, light-induced firing of action potentials has been observed in basal retinal neurons in primary cell culture during whole-cell patch-clamp recordings (S. Michel, unpublished observations). Light responses have also been observed in dispersed *A. californica* retinal cells maintained in culture; these cells appear to be homologous in many ways to the basal retinal neurons (Jacklet and Barnes 1993). It is not yet clear what part of the transduction process is sometimes lost from basal retinal neurons maintained in culture, although the cells may be unable to recover from photobleaching generated during dispersal.

One well-studied circadian system, the avian pineal gland, has been shown to contain opsin and many of the molecular components of the light transduction mechanism of vertebrate retinal photoreceptors (reviewed by Foster et al. 1989; Takahashi et al. 1989). However, the circadian pacemaker of the avian pineal gland may be entrained through an unusual transduction process, since pertussis toxin, which is known to block transducin, blocks the acute light response of the pinealocytes but does not block phase shifts in response to light pulses (Zatz and Mullen 1988). In addition, phase shifts are not blocked by prior depletion of carotenoids, whereas acute light responses are inhibited (Zatz 1994). Because the light activation of G proteins and second messengers within the basal retinal neurons has not been thoroughly investigated, this transduction process may still involve mechanisms unique to circadian systems.

An obvious feature that distinguishes both the basal retinal neurons and the avian pinealocytes from photoreceptors of the visual system is the lack of spatial separation of the photoreceptive membrane from the rest of the cell. In the basal retinal neurons, only the final step of the transduction pathway, viz. depolarization and subsequent movement of calcium into the cell, appears to result in the phase shift (reviewed by Geusz and Block 1994). If calcium entry is prevented, light still causes a depolarization but the phase shift does not occur (McMahon and Block 1987; Khalsa and Block 1988). If, as the present study indicates, photon capture takes place within the basal retinal neurons, the cell must be able to separate, spatially or otherwise, the early biochemical events of the transduction process from the molecular components of the circadian pacemaker. How this is accomplished should be revealed as more is learned about the pacemaker and its entrainment pathway.

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