Photoreception of Paramecium cilia: localization of photosensitivity and binding with anti-frog-rhodopsin IgG

YASUO NAKAOKA*, RYO TOKIOKA
Department of Biophysical Engineering, Faculty of Engineering Science, Osaka University, Toyonaka, Osaka 560, Japan

TAKAO SHINOZAWA, JUN FUJITA
Department of Biological and Chemical Engineering, Faculty of Engineering, Gunma University, Kiryu, Gunma 376, Japan

and JIRO USUKURA
Department of Anatomy, Nagoya University, School of Medicine, Nagoya 466, Japan

* Author for correspondence

Summary

Paramecium bursaria is photosensitive and accumulates in a lighted area. The cells can be deciliated by a brief suspension in dilute ethanol. Both intact and deciliated cells showed depolarization in response to light stimulation by a step-increase from dark to above 0.7 mW cm⁻² (550 nm). On the other hand, after a step-increase to below 0.4 mW cm⁻², intact cells showed hyperpolarization, while the deciliated cells showed no change in membrane potential. This difference in membrane potential response between ciliated and deciliated cells suggests that both somatic and ciliary structures are photosensitive. In our search for the photoreceptive molecules, a polyclonal antibody induced in rabbits against frog rhodopsin was found to cross-react with a 63 x 10³ Mr protein of P. bursaria, by immunoelectrophoresis. Immunocytochemical studies showed that the antibody labeling was localized on both the ciliary and the somatic membranes. These results raise the possibility that P. bursaria may contain a rhodopsin-like protein as a photoreceptor molecule.

Key words: photoreceptor, cilia, rhodopsin, immunocytochemistry, Paramecium bursaria.

Introduction

Paramecium bursaria is photosensitive and accumulates in an area of appropriate light intensity (Engelmann, 1882; Matsuoka and Nakaoka, 1988). In correspondence with such accumulation, the cells show an avoiding response to both a step-increase to strong light and a step-decrease from an appropriate intensity of light (Cronkite and Van Den Brink, 1981; Iwatsuki and Naitoh, 1981; Saji and Osawa, 1974). Matsuoka and Nakaoka (1988) have recorded a photoreceptor potential that shows a steady depolarization after a step-increase in the light intensity and recovery to the resting level upon a step-decrease. The action spectrum of the depolarization has two peaks at 420 nm and 560 nm. It has been shown that the depolarization is elicited by a local stimulation to the anteroventral portion of the deciliated cell (Nakaoka, 1989). Such depolarization causes the avoiding reaction in response to strong light, because the membrane depolarization triggers action potentials that are evoked on the ciliary membrane (Dunlap, 1977; Ogura and Takahashi, 1976). The avoiding reaction in response to a step-decrease in light intensity, however, is inconsistent with the corresponding potential change, which repolarizes to the resting level. To solve such inconsistency, the potential changes in response to light stimulation of low intensity were recorded in this study. Comparison of the potential responses between intact and deciliated cells suggested that the cilia are also photosensitive.

Tokioka et al. (1990) found that P. bursaria contains retinal, which is known to be a chromophore of the visual pigment. So, we assumed in this study that rhodopsin might be the photoreceptive molecule as in higher organisms with retinas, and we applied immunocytochemical methods using an antibody against frog rhodopsin. The reactive regions of the cell were studied in relation to the results of electrophysiological measurements.

Materials and methods

Cells

Chlorella-containing Paramecium bursaria (stock BND-1G and OK-1, supplied by Dr I. Miwa of Ibaraki University) and Chlorella-free P. bursaria (stock CT-12W) were cultured in a hay infusion inoculated with Klebsiella pneumoniae. The cultures were maintained under a fixed illumination cycle of 12 h light (a fluorescent light of about 10³ lx) and 12 h dark at 25°C. Stationary-phase P. bursaria were collected by low-speed centrifugation and suspended in an experimental solution containing 1 mM CaCl₂, 0.5 mM MgCl₂, 4 mM KCl and 2 mM Tris-Cl (pH 7.2) for 1–3 days under the illumination cycle.
Deciliation

In some instances of intracellular recordings, the cells were deciliated by incubation in the experimental solution containing 5% ethanol for 2-3 min at 22°C and then returned to the experimental solution (Nakaoka et al. 1987); this procedure is similar to that of Ogura and Machemer (1980).

Intracellular recording

Methods of intracellular recording were similar to those described in a previous report (Matsuoka and Nakaoka, 1988). The electrodes were filled with 0.1 M KCl, and their resistances were 100-150 MΩ. The cells were placed in a glass vessel mounted on an inverted microscope (Olympus, IMT-2), and two electrodes were inserted from the upper side, one for recording potential and one for injection of current. The external solution was the experimental solution, with its temperature controlled at 25°C by water flow beneath the vessel.

Light stimuli

A 50 W halogen lamp, which was the light source for the inverted microscope, was also used as the source for stimulation. The light intensity was controlled by d.c. variable-supply voltage. An interference filter (550 nm, a half-bandwith of 10 nm) was placed in front of the lamp to obtain monochromatic light. Pulses of light were obtained by using a shutter placed between the condenser diaphragm and the condenser lens, and were recorded with a photodetector placed on one of the eyepieces. The light intensity was measured using a calibrated silicon photodiode.

Preparation of antibodies

Rabbit IgG was prepared by the injection of the frog rhodopsin fraction resulting from SDS-PAGE into rabbits subcutaneously, and the IgG was purified by DEAE-cellulose column chromatography, as described (Shinohara et al. 1987).

Protein electrophoresis

P. bursaria cells were disrupted in 0.1 M HCl. After neutralization, in order to remove chlorophyll, the disrupted cells were washed five times with 80% acetone according to the procedure described by Morrisey et al. (1989). Rod outer segment (ROS) proteins were prepared as previously described (Shinohara et al. 1987). Proteins of ROS and P. bursaria were solubilized in the SDS sample solution (7% SDS, 70 mM dithiothreitol, 20 mM EDTA and 0.06% BPP). The mixture was boiled for 3 min. Separation by SDS-PAGE was done on a 15% polyacrylamide resolving gel and a 5% polyacrylamide stacking gel.

The amount of protein was determined by Lowry's method using BSA as a standard. Molecular weight standards were obtained from Pharmacia (Sweden).

Western blotting

Separated gels were transferred electrophoretically to nitrocellulose paper with a Heraeus Blot (model AE6E70, Atto) in a blotting buffer (100 mM Tris–HCl, 192 mM glycine, 20% methanol and 0.1% SDS, pH 8.8). The blot was cut into a number of identical strips. The polypeptide bands on one strip were stained with CBB (Comassie Brilliant Blue R-250). The remaining strips were shaken for 1 h in TBSA (50 mM Tris–HCl, 150 mM NaCl and 5% BSA, pH 7.5) to eliminate nonspecific protein binding and were incubated with anti-frog-rhodopsin rabbit IgG (1.9 µg ml−1) in TBSA for 1 h. To remove nonspecific binding of IgG, the strips were vigorously shaken once in TBSA supplemented with 0.1% Tween-20 and 0.1% SDS for 5 min and washed five more times in a solution containing 50 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20 and 0.1% SDS. Blot-strips were incubated in horse-radish peroxidase-labeled goat anti-rabbit IgG (30 µg ml−1, Capel Laboratories Inc) in TBSA.

Immunofluorescence microscopy

Chlorella-free P. bursaria was used for fluorescence observation. The cells were washed three times in Dryl's solution using low-speed centrifugation, and fixed in 4% paraformaldehyde in a 10 mM phosphate-buffer (pH 7.3) for about 12 h. After washing the cells three times in the phosphate buffer, using centrifugation, the cells were incubated for 30 min at room temperature in homologous goat serum in phosphate buffer containing 4% bovine serum albumin (BSA), then washed in phosphate buffer. After washing, the cells were incubated in Rabbit antisera against frog rhodopsin (dilution 1/30, concentration of IgG was about 2 µM) or in homologous rabbit antisera as a control in 10 mM phosphate buffer containing 1% BSA. After several rinses in phosphate buffer, the second FITC-conjugated goat anti-rabbit IgG was added for 1 h at room temperature. After washing three times in phosphate buffer the cells were observed using a fluorescence microscope (Nikon, Fluophot). Photographs were taken using a color film (Fuji ASA 1600).

Immunoelectron microscopy

P. bursaria were collected from culture media by gentle centrifugation, and the cells were immersed directly in fixative consisting of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at room temperature. They were then washed a few times with the same buffer. Specimens were dehydrated with an ascending series of ethanol and infiltrated with LR White. Thin sections were mounted on 100 mesh grids covered with Formvar. Immunocytochemical labeling with anti-rhodopsin antibody was achieved on thin sections using a two-stage method as described previously (Usukura and Bok, 1987). Goat anti-rabbit IgG absorbed to 10 nm colloidal gold was employed for visualizing binding sites.

Results

Photoresponse potential

Potential response upon weak light stimulation was different between ciliated and deciliated cells (Fig. 1). When the light intensity for stimulation was lower than 0.4 mW cm−2, a step-increase in the light intensity induced hyperpolarization in ciliated cells, but induced no change in the membrane potential of deciliated cells. A step-decrease caused membrane depolarization of the ciliated cells, which was accompanied by frequent action potentials. When the light intensity was higher than 0.7 mW cm−2, the step-increase induced a depolarization and step-decrease resulted in recovery of the potential to the level before stimulation. The potential response of the ciliated cells was similar to that of the deciliated cells except that the former generated frequent action potentials upon depolarization caused by the step-increase in light intensity.

The amplitudes of the potential change in response to step-increase are summarized as a function of the light intensity in Fig. 2. As described above, the differences in the potential response between ciliated and deciliated cells are found at lower intensity (below 0.4 mW cm−2), while at higher intensity (above 1.6 mW cm−2) the amplitudes of the depolarizing receptor potential of ciliated cells are similar to those of deciliated cells. At intermediate intensity between these the potential response of the ciliated cells was either hyperpolarization or depolarization.

Voltage-dependence of the receptor potential

To discover whether the membrane conductances generating either the hyperpolarizing or the depolarizing response are the same or different, reversal potentials for these potential responses were sought by injection of a constant current (Fig. 3). In ciliated cells, the hyperpolar-
Fig. 1. Potential changes of intact (A) and deciliated (B) cells in response to step-changes in the graded intensity of light. 10-s pulses of 650 nm light were repeatedly applied as shown below the recordings of potential ('up' means on).

Light intensity (mW cm\(^{-2}\))

<table>
<thead>
<tr>
<th>Light intensity (mW cm(^{-2}))</th>
<th>0.14</th>
<th>0.4</th>
<th>0.7</th>
<th>1.6</th>
<th>5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Deciliated</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 2. Relationship between the amplitude of the potential response (ΔV) and light intensity. Potential changes of intact (○) and deciliated (●) cells were measured at the onset of 10-s light pulses of various intensities. Each point is the mean of 5 different experiments. Vertical lines show half standard deviations.

Light intensity (mW cm\(^{-2}\))

<table>
<thead>
<tr>
<th>Light intensity (mW cm(^{-2}))</th>
<th>0.05</th>
<th>0.5</th>
<th>1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Deciliated</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. Dependence of receptor potential upon potential shift. Resting potentials of intact (A) and deciliated (B) cells were shifted to various levels indicated by the values on the lefthand side; then, 10-s light pulses were repeatedly applied as shown below the potential recordings ('up' means on). Light intensities in A and B were controlled, respectively, at 0.4 mW cm\(^{-2}\) and 1.6 mW cm\(^{-2}\). Resting potential for A (~25 mV) and B (~23 mV) is indicated by 0.

Light intensity (mW cm\(^{-2}\))

<table>
<thead>
<tr>
<th>Light intensity (mW cm(^{-2}))</th>
<th>0.4</th>
<th>1.6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Membrane potential (mV)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. Deciliated</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Binding of anti-frog-rhodopsin IgG to P. bursaria proteins

Binding of anti-frog-rhodopsin rabbit IgG to P. bursaria proteins (Fig. 4, lanes A–C) was studied by Western blotting. The specificity of the IgG to frog rhodopsin was
Immunofluorescence
proteins (lane A-C; 50 μg/lane) were proteins. P. bursaria Paramecium proteins were solubilized at room tempera-
cells (Fig. 5A) was apparently brighter than that of cells reacted especially to cilia, and the fluorescence of whole symbiotic
The primary antisera proteins, the P. bursaria et al. (1987). Of the P. bursaria proteins, the anti-rhodopsin IgG was clearly bound to a 63 x 10^3 M,
confirmed by using frog ROS proteins, as reported (Shinozawa et al. 1987). Of the P. bursaria proteins, the anti-rhodopsin IgG was clearly bound to a 63 x 10^3 M,
Immunofluorescence
Visualization of rhodopsin-like molecules by indirect immunofluorescence was done by using Chlorella-free cells in order to avoid the fluorescence emitted by a large number of symbiotic Chlorella. The primary antisera reacted especially to cilia, and the fluorescence of whole cells (Fig. 5A) was apparently brighter than that of cells stained with pre-immune sera (Fig. 5B), whose fluorescence seems to have some non-specific origin. The fluorescence of cilia was usually brighter in the anterior part than in the posterior part.

Immunoelectron microscopy
In Paramecium there is one plasma membrane covering cilia and soma. The soma membrane covers subpellicular alveoles (Fig. 6). Anti-rhodopsin antibody binding sites were located exclusively at the surface membrane of the soma and cilia (Fig. 6A). Little labeling was observed on the membrane of subpellicular alveoles. As a control, treatment with pre-immune serum showed no significant labeling (Fig. 6B).

Discussion
Electrophysiological studies show that upon light stimulation by a step-increase to below 0.4 mW cm⁻² (550 nm), ciliated cells elicited a hyperpolarizing receptor potential, whereas deciliated cells elicited no receptor potentials. Such a difference between ciliated and deciliated cells suggests that the cilia may be photosensitive. In a previous study (Nakaoka, 1989), the hyperpolarizing receptor potential was not noticed, because the light intensity used for stimulation was higher than that used in the present study, on the erroneous assumption that the receptor potential is elicited only by strong light. Stimulation by weak light led us to find the hyperpolarizing receptor potential of ciliated, intact cells. Because the receptor potentials of Paramecium in response to mechanical (Ogura and Machemer, 1980) and thermal stimuli (Hennessey et al. 1983; Inoue and Nakaoka, 1990) are modified very little by deciliation, the receptors to these stimuli were thought to be localized on the somatic membrane of the cell. The present finding is the first demonstration in ciliates that the cilia themselves contain sensory receptors, comparable to sensory cells of multicellular organisms including photoreceptors, rods and cones (Vinnikov, 1965).

With light stimulation consisting of a step-increase to above 0.7 mW cm⁻², both ciliated and deciliated Paramecium elicited depolarization receptor potentials of similar amplitude, which is in agreement with previous results (Nakaoka, 1989) and confirms that the deciliated cell is photosensitive. The presence of both hyperpolarizing and depolarizing receptor potentials is consistent with the photophobic behaviour; the phobic responses are induced either by a step-decrease from a low intensity or by a step-increase to a strong intensity, and both changes in the light intensity cause membrane depolarizations that trigger action potentials.

Since the reversal potentials of the hyperpolarizing and the depolarizing receptor potentials are present, respectively, at negative and positive levels from the resting potential, these receptor potentials having opposite polarities are elicited by changes in the membrane conductances for different ionic species. The depolarizing receptor potential has been shown to be induced by a change in membrane conductance for Ca²⁺ (Nakaoka et al. 1987). The ionic species of the membrane conductance that induce the hyperpolarizing receptor potential has not yet been determined. Increase in the light intensity used for stimulation causes transformation of the receptor potentials from hyperpolarization to depolarization. This might be caused by a change in the membrane impedance eliciting each type of receptor potential, although this need to be studied further.

The receptor pigments in the eyes of all multicellular animals contain rhodopsin as the chromophore. In a unicellular alga, Chlamydomonas, it has been shown that retinal functions as the photoreceptive chromophore (Foster et al. 1984), and there are some light-sensitive pigments in the colored ciliates (Song, 1983; Finlay and
Fig. 5. Fluorescence micrographs of *P. bursaria* stained with primary anti-frog-rhodopsin followed by FITC-conjugated goat anti-rabbit IgG. (A) Stained with rabbit anti-frog-rhodopsin; (B) stained with homologous rabbit IgG as a control. Cilia at the anterior part have reacted strongly. ×550.
Fig. 6. EM immunocytochemical localization of anti-rhodopsin in *P. bursaria*. Sections were labeled with either anti-rhodopsin antibodies (A) or pre-immune control serum (B). Antibody binding sites were visualized by 10-nm gold particles conjugated with secondary antibody. The gold labels are distributed on the surface membranes of soma and cilia. Bars, 1 μm.

Fenchel, 1986). From the colorless ciliate, *P. bursaria*, Tokioka et al. (1990) have extracted retinal. The present finding that anti-rhodopsin antibody against frog rhodopsin specifically binds to a 63×10^3 M_d polypeptide of *P. bursaria* suggests the presence of a rhodopsin-like protein in the surface membrane of this cell.

Immunocytochemical studies to determine the site of reaction against rhodopsin antibody showed that the antibody reacts with both the ciliary and the soma membranes. In combination with the electrophysiological results showing that photosensitivity is localized on the soma and the cilia, the findings suggest that a rhodopsin-like protein may function as the photosensitive molecule in *P. bursaria*. If this view is established by more direct evidence, *P. bursaria* may become a unique model for studying photoreception and its evolution.

This work was supported by grants-in-aid from the Ministry of Education, Science and Culture of Japan (02680210), and
partially carried out under the NIBB Cooperative Research Program for the Okazaki Large Spectrograph (90-505).

References


(Received 9 November 1990 – Accepted, in revised form, 12 February 1991)