Light-induced Structural Changes of Cytoskeleton in Squid Photoreceptor Microvilli Detected by Rapid-freeze Method

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Abstract. The cytoskeleton in squid photoreceptor microvilli was studied by freeze-substitution electron microscopy combined with rapid freezing using liquid helium, under dark-adapted and light-illuminated conditions. In the dark-adapted microvilli, actin filaments were regularly associated with granular structures on their surface; these granular structures were cross-linked to the rhodopsin-bearing plasma membranes through slender strands. Upon exposure to light, the granular components detached from the actin filaments, which then appeared to be fragmented and/or depolymerized. These observations have led us to conclude that light stimulation triggers the breakdown of the microvillar actin filament complex in squid photoreceptor cells. The results are discussed with special reference to the physiological role of actin filaments in photoreception.

Materials and Methods

Preparation of Squid Retina for Rapid Freezing

Squid, Doryteuthis bleekeri, were kept alive in a small, circular, closed-system aquarium (1). To obtain the images of dark-adapted retinas, the squid was kept in complete darkness overnight in the aquarium and the eyeball was excised under infrared light. After the lens was resected with fine forceps, the retina was carefully cut into small pieces (~6 × 6 mm) using a sharp razor blade, so as not to touch the photoreceptive surface for the rapid freezing. All procedures from the dissection of the eyeball to the rapid freezing were performed under infrared light.

To obtain the images of light-illuminated retinas, a dark-adapted squid was illuminated with light for 5 min before dissection of the eyeball. Then the retina was resected, cut into small pieces, and rapidly frozen under light.

10 retinas from different eyeballs were examined in either dark-adapted or light-illuminated conditions.

Rapid Freezing

A trimmed, dark-adapted retina was placed on the specimen holder and rapidly frozen by slamming the photoreceptive surface against a pure copper block cooled to 4 K by liquid helium under infrared light. The light-illuminated retina was rapidly frozen under light.

IN both vertebrate and invertebrate photoreceptor cells, light stimulus is transduced into electric signals. The possible participation of the cytoskeleton in the photoreceptive process has attracted increasing interest. For analyzing this problem, the retinas of invertebrates offer an advantageous model because in most invertebrates, the rhodopsin-bearing photoreceptor plasma membranes are specialized into rhabdomeral microvilli with underlying cytoskeleton (4, 21).

In early studies by Zonana (21) and Cohen (4), some densities were recognized inside the squid photoreceptor microvilli. Varela and Porter (20) identified an axial filament in the axis of bee photoreceptive microvillus. Some subsequent studies on invertebrate retinas also described the filamentous axial densities, which were occasionally cross-linked with the membrane by slender strands—"side-arms" (2, 6, 13).

The chemical nature of the microvillar cytoskeleton of photoreceptor cells was also studied using the isolated rhabdomeral microvilli fraction (6, 13). The findings obtained favored the notion that the microvillar cytoskeleton (the axial filament and side-arms complex) was mainly composed of actin and its associated proteins. Throughout these studies, the investigators arrived at a consensus: in invertebrate photoreceptors, the microvillar cytoskeleton was so labile that it was very difficult to preserve its ultrastructural integrity by conventional chemical fixation. Therefore, at the electron microscopic level, it was thought impossible to analyze the dynamic aspects of the microvillar cytoskeleton induced by light stimulation. It is now widely accepted that the freeze-substitution technique aided by rapid freezing is highly potent for analyzing the ultrastructure of the labile and/or dynamic structures (17, 18). The present study took advantage of applying the rapid-freezing method on the squid photoreceptive microvilli for electron microscopic observations. Here we describe that (a) a dark-adapted microvillus is characterized unequivocally by an axial filament which is regularly associated with the granular components and cross-linked to the membrane through the slender strands, and (b) light illumination of the microvillus induces the dissociation of granular components from the axial filaments and also the fragmentation of the axial filaments.
Figure 2. Transverse-sectional images of rhabdomeral microvilli in dark-adapted (a) and light-illuminated (b) retinas. (a) Each dark-adapted microvillus presents only one dark dot of 10-15 nm diam in its center (arrows). (b) Upon exposure to light, the central dot seen in a disappears and several smaller dots of ~5 nm diam are seen scattered in each microvillus (arrows). The structural changes detected by rapid-freezing and freeze-substitution methods were highly reproducible and the freeze-substitution images showed no variability of the microvillar cytoskeleton within each section. Bar, 0.1 μm.

**Freeze-substitution and Freeze-etch Replica Electron Microscopy**

The frozen retinas were freeze substituted for 2 d with absolute acetone containing 2% OsO₄ at -80°C (17, 18). The samples were slowly warmed by transfer to a -20°C freezer for 1 h, and then kept at 4°C for 2 h. After being washed twice in absolute ethanol at room temperature, the samples were stained en bloc in absolute ethanol containing 5% uranyl acetate at room temperature for 2 h. They were then washed sequentially in absolute ethanol and in propylene oxide, and embedded in Epon 812. Thin sections were doubly stained with uranyl acetate and lead citrate for 3 min each, and then examined in a JEOL 1200-EX electron microscope operated at 100 kV. The freeze-etch replica method used in this study was previously described elsewhere (19).

**Optical Diffraction**

For the optical diffraction analysis of the single axial filaments in the microvilli, we used longitudinal thin sections (500-700 Å thick). A rectangular mask was overprinted on the electron micrographs (see reference 17).

**Results**

**Overall Structure of Freeze-substituted Retina**

In the freeze-substituted samples of the squid retina, thin sections cut perpendicular to the metal contact surface revealed that the superficial layer 10-20 μm in depth was ultrastructurally well preserved under both dark-adapted and light-illuminated conditions. In this region, the well-formed, straight microvilli on the photosensory cell processes formed rhabdomeres (Fig. 1). Since we mainly used the distal layer of retina for rapid-freeze study, the rhabdom structures of microvilli were somewhat dispersed in their array. In the freeze-substituted specimens, the microvillar cytoskeleton was reproducibly well preserved. 10 retinas of different eyeballs were examined for the analysis of microvillar cytoskeleton, in either dark-adapted or light-illuminated conditions.
Transverse-sectional Views of Microvilli

The trilaminal cell membrane was clearly identified in each transversely sectioned microvillus. The diameter of the light-illuminated microvillus was seen to be an average of 5-10% larger than that of dark-adapted microvilli which showed uniform diameter of 50 nm. Though the degree of swelling differed among the microvilli in the light-illuminated retinas, the light-induced changes of microvillus cytoskeleton (as described below) were detected in every case. In either dark-adapted or light-illuminated conditions, the extracellular matrix appeared to link microvilli with one another (Fig. 2).

When the transverse-sectional profiles of the microvilli in dark-adapted retinas were compared with those in light-illuminated retinas, significant differences were discerned in the appearance of the cytoplasm. In dark adaptation, each microvillus in transverse section presented only one dark dot of 10-15 nm across in its center (Fig. 2 a). In rare cases, two dark central dots were distinct. Close inspection indicated that the dark dot appeared to be a complex of several smaller dots of ~5 nm across, each one connected to the membrane through slender strands ~15 nm in length. On the basis of the recent studies of chemically fixed retinas, it is most likely that the dark dots represent the transverse-sectional profiles of the axial filaments, which are thought to be actin-based structures. When exposed to light, this central dot disappeared and, instead, several smaller dots of ~5 nm across were seen to be scattered in the cytoplasm of each microvillus (Fig. 2 b).

Longitudinal-sectional Views of Microvilli

The images of longitudinally sectioned microvilli were most helpful in interpreting the structural changes of cytoskeletons induced by light illumination. When a microvillus in the dark-adapted retina was longitudinally cut through its axis, a single axial filament (in rare cases two axial filaments) ~15 nm thick was clearly observed to run the full length of each microvillus from the tip to the base (Figs. 3 a, 4, and 6, a-c). Close inspection revealed that this axial filament was covered on its surface with granular structures whose diameter was measured to be ~5 nm. In the optical diffraction patterns obtained from longitudinally sectioned single axial filaments, the layer line with an axial spacing of 37 nm (the crossover repeat of actin filament helix) was obtained (Fig. 4 c). As shown in our previous study, this axial spacing was not detected from the nondecorated actin filaments embedded in Epon; and the layer line of this spacing can be clearly observed only when actin filaments are regularly decorated with some large molecules, such as heavy meromyosin (17). Taken together with recent studies of the biochemical nature of axial filaments (6, 13), it is safe to say that in the dark-adapted microvillus an actin filament is regularly decorated along its helix with granular structures, forming an axial filament. It is noteworthy that the axial filaments were seen to be connected to the plasma membranes through slender strands, ~15 nm long.

In sharp contrast, when the dark-adapted retinas were illuminated, the distinct axial filaments were not visible in almost all microvilli. Instead, in the cytoplasm of the microvilli were scattered granular structures of ~5 nm thick, slender strands, and smooth-surfaced, short, thin filaments 5 nm thick with an actin-like appearance (Figs. 3 b, 5, and 6, d-f) which exhibit no layer line in their optical diffraction patterns (Fig. 5 c). These observations lead us to conclude that upon exposure to light the axial filaments may be broken down, and that the fragmentation of the actin filaments and the dissociation of the granular structures occur simultaneously.

The freeze-etch replica images of the microvillar cytoskeleton further substantiated our results from the thin-sectional images of freeze-substituted microvilli, though the cytoplasm of microvilli was only rarely exposed by freeze fracturing due to their small diameter. In the dark-adapted microvilli, each axial filament showed granular structures on its surface being connected with the plasma membrane through slender strands (Fig. 4, d and e). Under light-illuminated conditions, the granular structures and slender strands were observed to be scattered in the cytoplasm of microvilli. Occasionally short thin filaments were discerned. These filaments were characterized as the nondecorated actin filaments by the 5-nm periods on their surfaces (Fig. 5, d and e).

Oblique-sectional Views of Microvilli

Based solely on the longitudinally sectioned images of microvilli, it is difficult to say whether the axial filaments are really fragmented by light illumination; we can not exclude the possibility that the continuous axial filaments in the light-illuminated microvilli may snake in and out of the plane of the section, appearing to be discontinuous. To check this point the oblique-sectional images of microvilli were most informative. In dark-adapted retinas, every obliquely cut microvillus displayed one (or two) axial filaments, indicating that the axial filaments were continuous throughout the full length of the microvilli (Figs. 3 a and 4). Distinct axial filaments along the microvillar axes were not observable in the obliquely cut microvilli (Figs. 3 b and 5), under the light-illuminated condition. The granular structures and slender strands were scattered in the microvillar cytoplasm. A few microvilli displayed thin filaments, ~5 nm across, with an actin-like appearance in light-illuminated retinas.

Transition between Dark-adapted and Light-illuminated States of Photoreceptive Microvillar Cytoskeleton

The results obtained are shown schematically in Fig. 7; the cytoskeleton in the photoreceptive microvilli can be found in

Figure 3. Longitudinal- and oblique-sectional images of photoreceptive microvilli of the dark-adapted (a) and light-illuminated (b) retinas. (a) In the dark-adapted microvillus, a single axial filament is discerned in longitudinally sectioned microvilli (large arrows). Note that each dark-adapted microvillus represents an axial filament as revealed in oblique sections (small arrows). (b) The continuous axial filaments are hardly observed in the longitudinally sectioned microvilli under light-illuminated conditions (large arrows). Typical axial filaments are barely observable in obliquely cut microvilli (small arrows). Bar, 0.1 μm.
at least two different states, dark-adapted and light-illuminated. In dark-adapted microvilli, actin filaments are regularly associated with granular structures on their surface (thus forming axial filaments) and cross-linked to the rhodopsin-bearing plasma membranes through the slender strands. Upon exposure to light, the granular components detach from the actin filaments, which are possibly fragmented and/or depolymerized. This structural change of the microvillar cytoskeleton induced by light illumination was also observed when the dark-adapted retinas were resected before light illumination (data not shown).

The transition between these two states was bidirectional. For example, when dark-adapted retinas were illuminated for 10 min, resected, and then the retinal slices were placed in complete darkness again for 5 min, the microvillar cytoskeleton showed a typical dark-adapted state (data not shown).

One point to be examined is whether the light-induced structural change of the microvillar cytoskeleton reported here is limited to the distal layer of the retina (since only the distal layer was able to be observed in our freezing method). For this purpose, we prepared retinal strips ~100 μm thick by cutting the retina parallel with the long axes of receptor cells, and froze the strips by slamming the newly exposed side against the cooled copper block. Though some parts of the retinal cells might be inevitably damaged in this preparation, the various depths of the retinal layer were expected to be rapidly frozen. The microvilli differed in their arrangements depending on the level of the photoreceptive cell processes in the retinas. In the distal layer, ~30 μm in depth, the rhodobin structures of microvilli were somewhat dispersed in their arrays, while in the proximal region the rhodobin structures were characterized by closely packed, hexagonal arrays such as those seen in the glutaraldehyde-fixed materials (4, 15, 20, 21). In the proximal layer, as well as the distal layer, the microvillar cytoskeleton tended to show the light-induced transition from the dark-adapted to the light-illuminated types (see insets in Figs. 4 b and 5 b), although the cytoskeletal organization of some microvilli could not be clearly discerned in this preparation, mainly due to mechanical damage.

Discussion

Taking advantage of the freeze-substitution method, combined with rapid freezing, for electron microscopic observation, we have succeeded in visualizing reproducibly the microvillar cytoskeleton in situ in squid retina. We have also demonstrated that the microvillar cytoskeleton takes two different states, dark adapted and light illuminated. In this study, due mainly to the technical limitations of the freezing method, only the distal region of retinal cells was analyzed.

Taken together with the classic work by Hagins et al. (8), which demonstrated that the distal region of the photoreceptive processes of retinal cells was as functional as the proximal region, it is safe to say that the light-induced changes in microvillar cytoskeleton reported here may be physiological and occur in the proximal region as well. This point was partially confirmed in the present study using strips of retinas.

It has been repeatedly said that the photoreceptive microvillar cytoskeleton is a labile structure, accessible to the damaging effects of chemical fixatives. Some chemical reagents though, such as Ca2+ chelators, thiol protease inhibitors, and calmodulin inhibitors, were shown to be effective in preserving the photoreceptive microvillar cytoskeleton (the complex of axial filaments and side-arms) (2, 6). In these studies, the light-adapted materials that were used and the images obtained were similar to the dark-adapted state of the cytoskeleton described in the present study. Therefore, it is possible that the chemical reagents modified the physiological state of photoreceptor cells before chemical fixation. In this connection, the intracellular Ca2+ levels (1, 3, 16) and the cGMP level (14) were shown to rise during light illumination in invertebrate photoreceptors. Recently, calmodulin was shown to be localized in the retinal microvilli (5). These observations lead us to imagine that the Ca2+/calmodulin system may be involved in the structural changes of microvillar cytoskeleton from the dark-adapted to light-illuminated type.

Evidence has accumulated that the microvillar cytoskeleton in retinal cells is actin based (6, 13). Our results on diffraction patterns obtained from the thin-sectional images of axial filaments support this finding. Recent studies on the isolated rhodobin microwill fraction suggested that a 145-kD protein was involved in the actin-based cytoskeleton (13). In the present study, two kinds of components other than actin filaments have been morphologically discerned in the microvillar cytoskeleton: the granular components and the slender strands. The granular components were arrayed along the helix of actin filaments under dark conditions, and were linked to the membranes through the slender strands, but were dissociated from the actin filaments upon exposure to light. Although we have no information about the chemical nature of these components, either component may be the morphological counterpart of the 145-kD protein. More recently, the genetic study on the Drosophila rhodomeres has led us to imagine that the microvillar cytoskeletal proteins (130 kD and 170 kD) are essential to light-evoked electrical

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**Figure 4.** High-power electron micrographs of dark-adapted photoreceptive microvilli. (a and b) Longitudinal- and oblique-sectioned microvilli, respectively. When the microvilli are nearly longitudinally cut through their axes, the axial filaments, which are 15 nm thick, are clearly observed to run the full length of the microvilli, which are covered with 5-nm-diam granular structures (arrowheads). Inset in b shows the proximal region of the retina rapidly frozen using the dark-adapted strips (see text). The axial filaments (arrows) are clearly discerned in microvilli which are tightly packed. (c) The optical diffraction patterns of the single axial filaments of dark-adapted microvilli. A layer line with the spacing of 370 Å (the crossover repeat of the actin filament helix) can be detected (arrows). (d and e) Freeze-etch replica images of the axial filaments in the microvilli. The axial filaments (arrowheads) are seen to be covered with the granular structures on their surface, which are cross-linked with the plasma membranes (m) through slender strands (arrows). Bars, 0.1 μm.
Figure 7. Schematic drawing of the microvillar cytoskeleton under dark-adapted and light-illuminated conditions. In the dark-adapted state, the axial filaments show regularly arrayed granular structures on their surface and the slender strands span the gap between the granular structures and the plasma membranes. Upon exposure to light, the granular structures and slender strands are scattered inside the cytoplasm of the microvilli.

Figure 6. Electron micrographs of the microvillar cytoskeleton in dark-adapted (a-c) and light-illuminated (d-f) retinas. (a-c) In the dark-adapted, photoreceptive microvilli, the axial filaments are seen to be covered on their surface with granular structures (arrowheads). Note the slender strands (arrows) which connect the axial filaments to the membranes. (d-f) In the light-illuminated retinas, the axial filaments are not distinct in the microvilli. The granular structures (arrowheads) and slender strands (arrows) appear to be scattered in the microvillar cytoplasm. Bar, 0.1 μm.

It was reported using chemical fixation that long-term light-illumination (for 1-2 d) induced the prominent vesiculation of microvilli as a turnover process in several kinds of invertebrates (7, 10). However, the studies on the squid retina to date have not arrived at a consensus about the turnover sites of the photoreceptive membranes. In this study, we used the 5-min light illumination to demonstrate the cytoskeletal organization of light-adapted microvilli and that vesiculation of microvilli did not occur. In our experiment, even light illumination for 2-3 h did not cause vesiculation of microvilli. Furthermore, the transition between dark-adapted and light-illuminated states of the microvillar cytoskeleton has been shown to be reversible. Taken as a whole the evidence indicates that the light-induced changes in microvillar cytoskeleton may not be coupled with the vesiculation of microvilli.

At present, it is difficult to conclude whether the light-induced structural changes of microvillar cytoskeleton play an important role in phototransduction, in the light-adaptation process, or in the initial stage of turnover of photoreceptive membranes. The freezing machine used in this study enables us to freeze the specimens only in a time course of minutes after light illumination. However, the phototransduction proceeds in a time course of milliseconds and the temporal resolution of the images obtained by the rapid-freezing technique itself is known to be at least 2 ms (9). Accordingly, to discern the physiological role (phototransduction, membrane turnover, or light adaptation) of the transition of the cytoskeletal organization reported here, it is necessary to develop a new freezing method which enables us to freeze the specimens within 10 ms after the beginning of light illumination. Studies along this line are now being conducted in our laboratory, and the preliminary results obtained appear to favor the notion that the microvillar cytoskeleton directly participates in the phototransduction process.

Figure 5. High-power electron micrographs of light-illuminated photoreceptive microvilli. a and b represent longitudinal- and oblique-sectioned microvilli, respectively. When the dark-adapted microvilli are illuminated, the distinct axial filaments are not visible in most microvilli. Instead, in the microvillar cytoplasm are scattered 5-nm-diam granular structures (thin arrows), slender rodlike structures (arrowheads), and short, thin filaments with an actin-like appearance (thick arrows). Inset in b depicts the proximal region of the retina rapidly frozen using the light-illuminated strips (see text). No axial filaments are clearly identified in all microvilli (asterisks) which are tightly packed. c The optical diffraction patterns of the single fragmented axial filaments under light-illuminated conditions. The thinfilaments in the microvilli exhibited no layer line. (d and e) Freeze-etch replica images of the light-illuminated microvilli. The distinct axial filaments are not observed in the microvilli. Only the granular structures and slender strands are seen to be scattered in microvillar cytoplasm. Note the short, actin-like, thin filaments with 5-nm periods on their surface (arrowheads). m, plasma membrane. Bar, 0.1 μm.

responses of the eye (12). To understand the molecular mechanism of the light-induced changes of microvillar cytoskeleton reported here, it is important to identify and characterize the structural components in the molecular term, paying special attention to their Ca²⁺-sensitive or -insensitive actin-binding ability and calmodulin-binding ability.

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