

Distinct Lobes of *Limulus* Ventral Photoreceptors

I. Functional and Anatomical Properties of Lobes Revealed by Removal of Glial Cells

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ABSTRACT Removing the glial cells that encase *Limulus* ventral photoreceptors allows direct observation of the cell surface. Light microscopy of denuded photoreceptors reveals a subdivision of the cell body into lobes. Often one lobe, but sometimes several, is relatively clear and translucent (the R lobes). The lobe adjacent to the axon (the A lobe) has a textured appearance. Scanning electron microscopy shows that microvilli cover the surface of R lobes and are absent from the surface of A lobes. When a dim spot of light is incident on the R lobe, the probability of evoking a single photon response is two to three orders of magnitude higher than when the same spot is incident on the A lobe. We conclude that the sensitivity of the cell to light is principally a function of the R lobe.

INTRODUCTION

The ventral photoreceptors of *Limulus* are a useful preparation for the study of the membrane conductances which generate the receptor potential (for a review see Fain and Lisman, 1981). Although the physiology of these cells has been extensively studied, there has been relatively little work on their anatomy. In the one major anatomical study (Clark et al., 1969), it was reported that ventral photoreceptors are surrounded by glial cells; that the photoreceptors contain microvilli typical of invertebrate visual cells; and that the microvilli are found in deep infoldings. Furthermore, regions of rhabdom (collections of tightly packed microvilli) were found to have no obvious compartmentalization within the cell.

In this paper we report a method for removing the glial cells and connective tissue surrounding ventral photoreceptors (henceforth termed "denuding").

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This method reveals for the first time that many ventral photoreceptors are subdivided into lobes that can be distinguished in the light microscope. Scanning electron microscopy shows that only one type of lobe has microvilli on its surface. Since the glial and connective tissue can be removed without destroying the ability of the photoreceptor to transduce light, it has also been possible to study the functional specializations of the lobes. We found that the sensitivity to light is, for the most part, restricted to the lobe bearing microvilli on its outer surface.

A preliminary report of some of these findings has appeared (Bacigalupo et al., 1981). Evidence is presented in a companion paper (Calman and Chamberlain, 1982) that the subdivision of ventral photoreceptors into lobes is not an artifact of the denuding process but is rather a typical feature of intact cells.

METHODS

Male *Limulus* (carapace 15–25 cm) were obtained from the Marine Biological Laboratory, Woods Hole, MA. Ventral nerves were removed from the animal under bright white light. Photoreceptors on the ventral nerve were individually denuded of their surrounding glia and connective tissue by the procedure described in Results. The suction pipette used to denude photoreceptors was fabricated as follows. Boralex micropipettes (100 μ l) were heated and pulled in a two-stage microelectrode puller in order to produce pipettes with a taper 3–6 mm in length. The tip was cut off by scoring the glass with sandpaper and then breaking off the end. Alternatively, the tips could be melted slightly by contact with a heated platinum filament. Upon cooling, the filament withdrew, producing a clean break in the glass. The tip (at this point 60–80 μ m in diameter) was then melted near a heated filament until it attained an inner tip diameter of \sim 20 μ m. The pipette was filled with artificial seawater (ASW) and placed in a micromanipulator controlled by a joystick. Surface material was removed from the cell by applying suction to the back of the pipette. During denuding, cells were observed through a compound microscope (150 \times magnification).

In preparation for scanning electron microscopy, denuded photoreceptors were fixed for 1 h in 2.5% glutaraldehyde. This solution was made by diluting 25% biological grade glutaraldehyde (Electron Microscopy Science, Fort Washington, PA) with concentrated ASW such that the final osmolarity of the solution after dilution was \sim 930 mosmol. The preparation was then washed in distilled water for several hours. To dehydrate the preparation, it was bathed for 5 min in each of the following alcohol solutions: 25, 50, 75, 90, and 95%, and three changes in 100% ethyl alcohol. The preparation was then critical-point dried from liquid CO₂ in a dryer following standard procedures. For viewing in the scanning electron microscope, specimens were attached to specimen mounts using double-stick tape. The specimens were sputter coated with gold palladium to give a coating thickness of \sim 200 Å, and they were viewed with a scanning electron microscope (SEM) at 20 kV.

To measure the responses of denuded cells to small spots of light, cells were held by a suction pipette and impaled with a conventional microelectrode. A spot was made by interposing a pinhole in a light beam. The pinhole was mounted on a X-Y micropositioner so that the spot could be moved. To position the spot without light-adapting the cell, we illuminated the preparation with infrared light and viewed the cell with an infrared image-converter attached to the microscope.

RESULTS

Denuding the Ventral Photoreceptor

The appearance of two ventral photoreceptors before and after the removal of glial cells and connective tissue is illustrated in Fig. 1. Before denuding, the cells bodies have no obvious substructure (Fig. 1*a*). After denuding, a subdivision of the cell into lobes is clearly apparent (Fig. 1*c*).

The method used for denuding cells is as follows: a ventral nerve is desheathed and pinned to a Sylgard substrate. Pronase (Calbiochem-Behring Corp., San Diego, CA; 20 mg/ml) is applied at room temperature until the tissue begins to show signs of loosening (~1 min). The pronase is removed and the tissue is then left undisturbed for 1 h in ASW. During this time some of the glial cells that surround the photoreceptor become round. A suction electrode is then used to pull on the connective tissue and glial cells to further loosen their connection with the photoreceptor. Then, starting at the axon hillock region, the glial cells and associated connective tissue are progressively peeled away. For this procedure to be successful, the photoreceptor must remain anchored to the ventral nerve by its axon. If the pronase treatment is too severe, the axon will easily detach from the ventral nerve and then there is nothing to hold the photoreceptor in place when suction is applied to the glial cells. If the denuding process has gone well, the cells will be firm and resistant to deformation. If the photoreceptors are soft and the cytoplasm appears to have liquified, gentler denuding is required. There is significant variability between animals in the ease of the denuding process.

The denuded ventral photoreceptors shown in Figs. 1*b-d* have two clearly distinguishable lobes. The lobe most distant from the axon appears somewhat translucent and clear; such lobes are termed R lobes. The lobes connected to the axon have more texture and are termed A lobes. This difference between R and A lobes is usually discernable though it is sometimes less obvious than in Figs. 1*b-d*. The basis for the choice of terminology R (rhabdomeric) and A (arhabdomeric) is given in the companion paper (Calman and Chamberlain, 1982).

The number, shape, and position of R lobes varies considerably from cell to cell, even within the same ventral nerve. Cells with one R lobe are more common along the ventral nerve than in the cluster of cells at the distal end of the nerve (the end organ). Some cells have two well-defined R lobes (see Fig. 4) and in the end organ, even more complex cells are occasionally seen. In cells with one R lobe, the R lobe is usually positioned opposite the axon (as in Figs. 1*b* and *c*), but sometimes the R lobe lies to the side of the A lobe (Fig. 1*d*). Cells with two R lobes are found rarely as isolated cells but usually as the distal member of a pair of closely opposed photoreceptors. The proximal photoreceptor typically has a single R lobe at its distal end that is juxtaposed to one of the R lobes of the distal cell. In pairs or clusters of photoreceptors, adjoining R lobes are strongly interconnected. To isolate a single cell from a cluster, the neighboring cells must be killed by breaking their membranes.

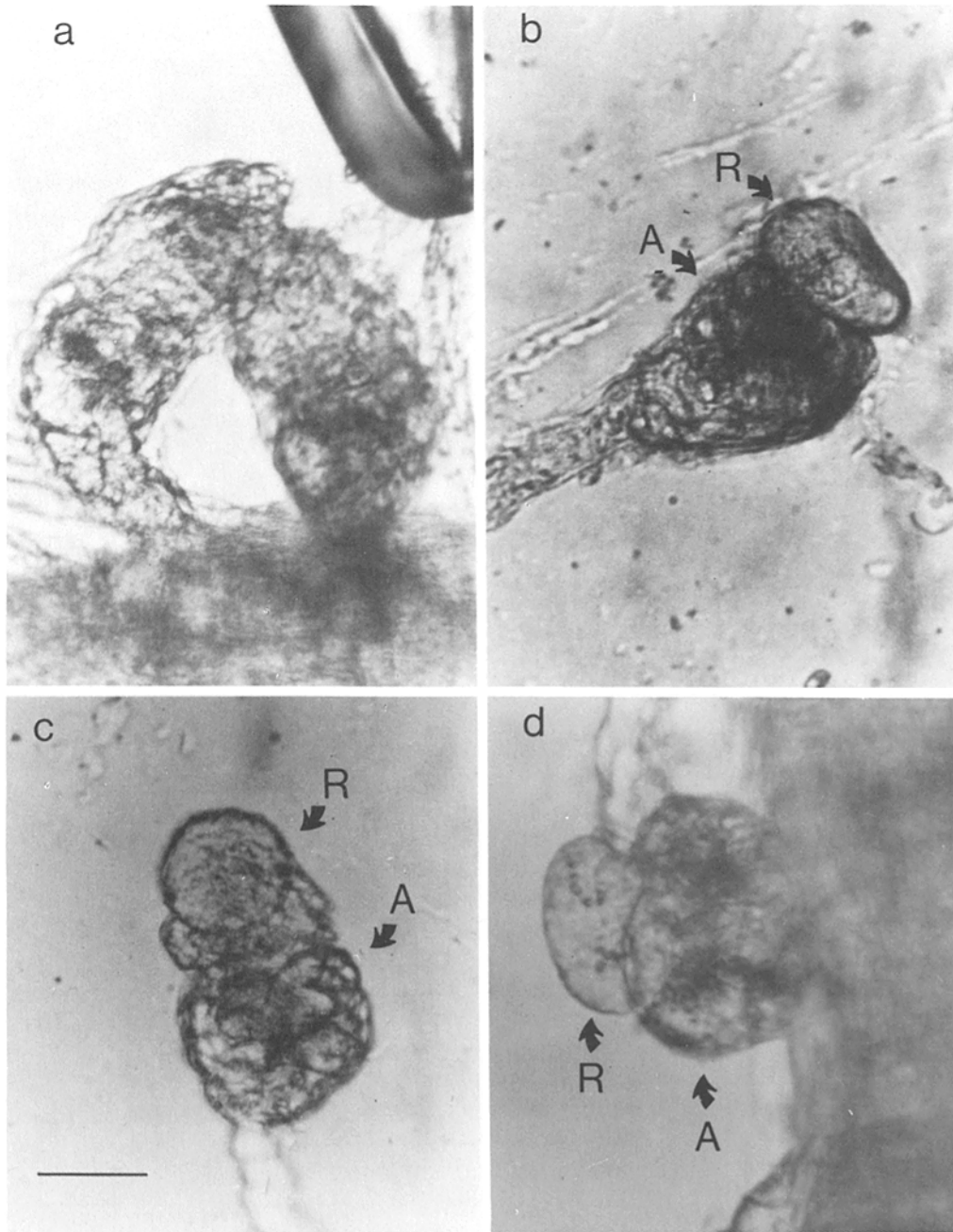


FIGURE 1. Appearance of ventral photoreceptors in the light microscope before and after removal of glia and connective tissue. (a) Two photoreceptors during an early stage of the denuding process. The cell bodies have been pulled away from the ventral nerve (bottom) but are still connected to it by their axons. The suction pipette used for removal of glial and connective tissue is seen at the top

Quite often what initially appears to be a single cell is seen to be a pair of cells after denuding.

In addition to the variability in the number and position of R lobes, there is considerable variation in their shape. Some R lobes are nearly spherical, as in Fig. 1*c*, some are conical, and still others form long finger-like projections. There is also variation in cell size. The teasing apart of end organs during the denuding process reveals a population of small cells, 15–25 μm in diameter. As judged by extracellular recordings using a suction electrode, at least some of the small cells generate receptor potentials (J. Stern, unpublished data).

Scanning Electron Microscopy of Ventral Photoreceptors

Cells were examined in the SEM using the procedure described in Methods. Fig. 2 shows a cell that has not been denuded. The cell is surrounded by a

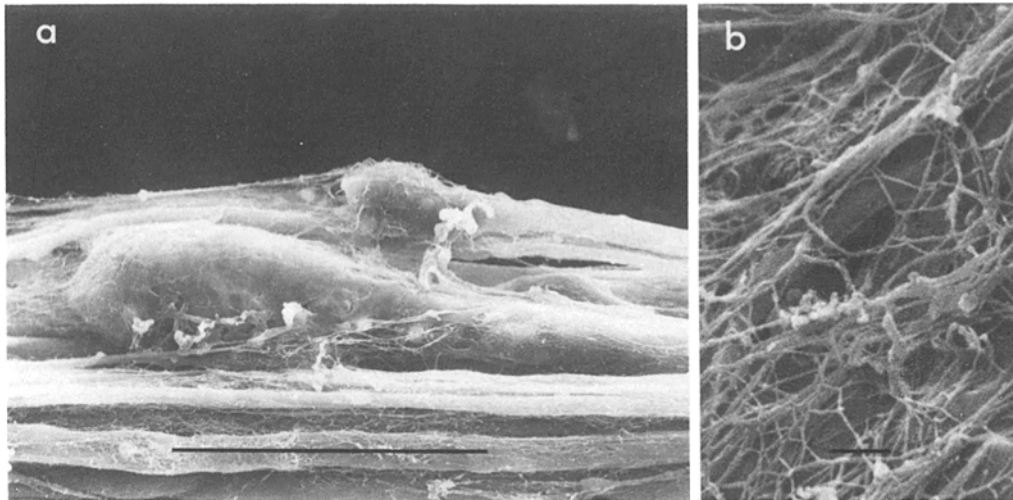


FIGURE 2. Appearance of cells in scanning electron microscope before denuding. (a) Photoreceptor embedded in the ventral nerve. Calibration bar 100 μm . (b) Closeup of fibers that cover glial cells. Calibration bar 1 μm .

fibrous material; lobes are not apparent. Fig. 3 shows a light micrograph of a denuded cell and a scanning electron micrograph of the same cell. The lobes visible in the light micrograph (Fig. 3*a*) are readily apparent in the scanning electron micrograph (Fig. 3*b*). The surfaces of the A and R lobes have a very different appearance, and at higher magnification (Fig. 3*c*) it can be seen that the surface of the R lobe is covered with microvilli, but none are seen on the A lobe surface.

of the photograph. (b) A cell after denuding. Note that there are two distinct lobes. (c) The cell in the right half of *a* after being denuded. (d) A denuded cell with the R lobe lying to the side of the A lobe of the cell. The cell's axon, which is out of focus, is attached to the upper left corner of the A lobe. Calibration bar in *c* is 50 μm and applies to the whole figure.

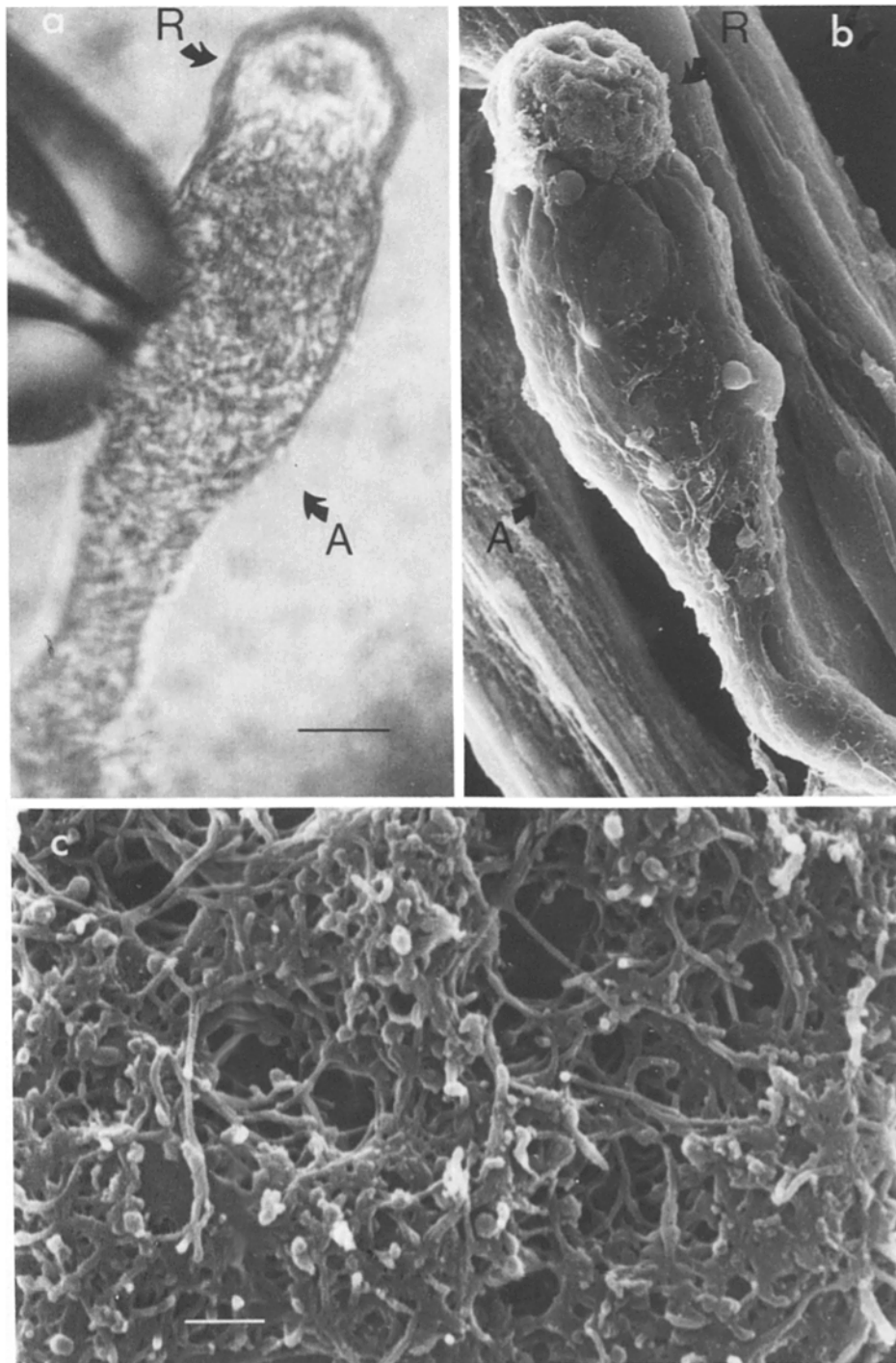


FIGURE 3. Appearance of denuded cell in the SEM. (a) Light micrograph of a denuded cell having one R lobe and one A lobe. Calibration bar 25 μm .

We have examined 12 denuded cells in the SEM. In six cells, microvilli were clearly visible. In one such cell there were two R lobes, both covered with microvilli (Fig. 4). In six cells, lobes were clearly distinguishable, but microvilli were not observed. It is possible that the lack of microvilli was caused by the denuding process or by the preparation for microscopy. There is often a well-defined cleft between R and A lobes (Fig. 4*d*) that forms the boundary between the microvillar R lobe and the smooth surface of the A

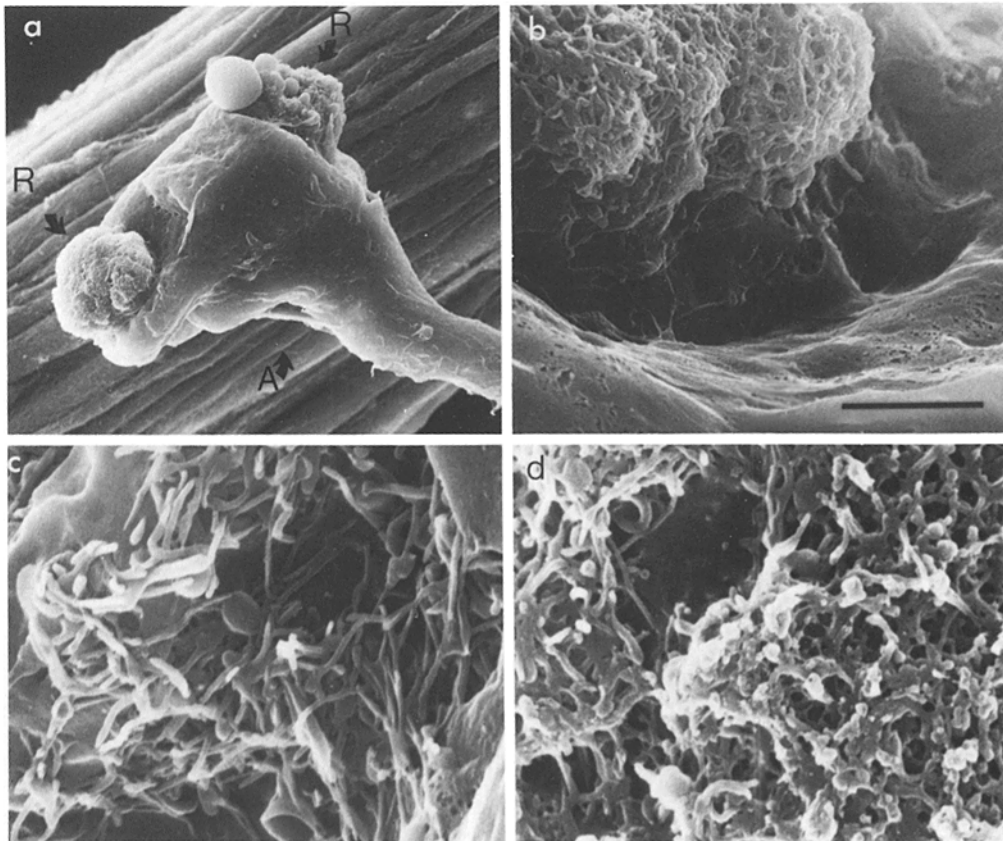


FIGURE 4. Scanning electron micrograph of a ventral photoreceptor having two R lobes and one A lobe. The two R lobes are located at the sides of the A lobe. The smooth bleb seen on the right R lobe was observed in the light microscope before fixation and probably resulted from local damage. (b) Close-up of the junction between the left R lobe and the A lobe, showing the invaginations of the membrane in this region. (c) Close-up of the left R lobe showing the microvilli. (d) Close-up of the left R lobe. A calibration bar is shown in *b* and represents 48 μm in *a*, 5 μm in *b*, and 2.2 μm in *c* and *d*.

(b) The same cell seen in the SEM. Note that after being denuded the isolated cell (seen in *a*) was placed on the surface of the ventral nerve. (c) Close-up of the R lobe showing microvilli. Calibration bar 1 μm .

lobe. Another interesting feature is dimplings of the R-lobe surface (Fig. 3*b*), which may be sites of membrane infoldings.

Sensitivity of the R Lobe and the A Lobe to Light

Fig. 5 illustrates an experiment in which we investigated the light sensitivity of the A and R lobes using a small spot of light (nominal diameter, 10 μm). The photograph shows a denuded photoreceptor held by a suction pipette and impaled by a microelectrode to record membrane potential. In all such

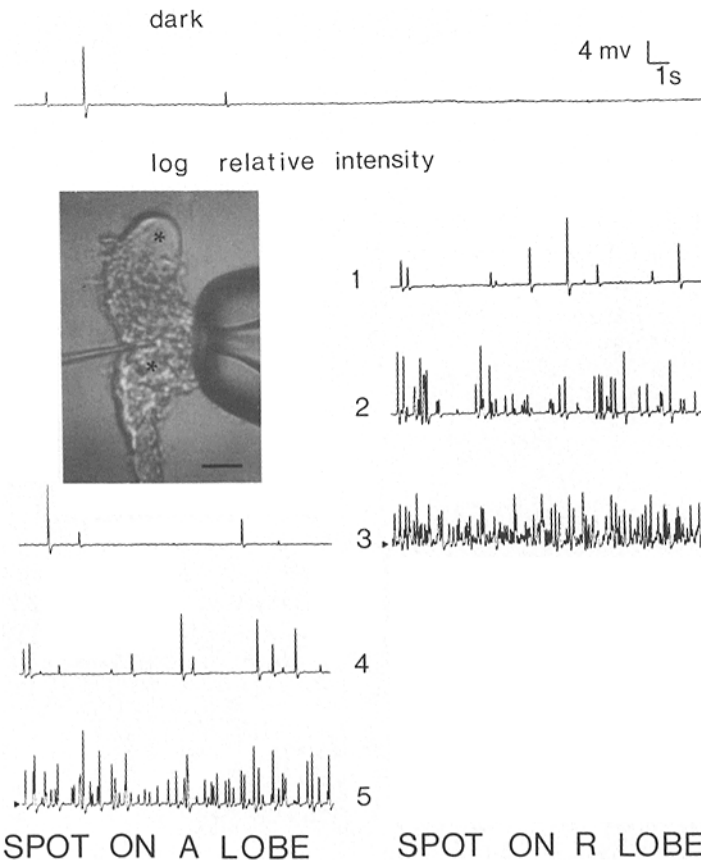


FIGURE 5. Differential sensitivity to light of the A and R lobes; inset shows denuded cell held by suction pipette and impaled by a microelectrode. The upper star marks the position of the small spot of light used to stimulate the R lobe. Similarly, a spot at the lower star was used to stimulate the A lobe. The calibration bar is 25 μm . The traces show changes of membrane voltage recorded by the microelectrode. The uppermost trace has three upward deflections, each of which is a quantum bump. It can be seen from the record that this cell had a low rate of quantum bumps in the dark. The remainder of the traces show the response to light for steady spots placed on the A lobe (left) or R lobe (right). The intensity of the spot was increased in 10-fold steps from top to bottom (log relative intensity is given by each trace; absolute intensity was not measured).

experiments the A lobe was impaled. Since the inside of ventral photoreceptors is isopotential (Brown et al., 1979), the potential recorded by the microelectrode is the same regardless of its location. The records show quantum bumps obtained under conditions of illumination which are indicated at the side of each trace. The dark rate is shown in the uppermost trace. When the A lobe was illuminated by a dim spot (designated "log intensity 3" in Fig. 5), the quantum bump rate was comparable to the dark rate: when the same spot was placed on the R lobe, it caused a dramatic increase in the quantum bump rate. Similarly, over a wide range of intensities, the response was greater when the spot was placed on the R lobe than when it was placed on the A lobe.

To quantify the difference in sensitivity between the two lobes, the rate of light-evoked quantum bumps was determined by subtracting the spontaneous rate from the total bump rate during illumination. Conditions were selected so that the bump rate was low enough to allow accurate identification of individual bumps. Since the light-evoked quantum bump rate is linearly related to light intensity (Fuortes and Yeandle, 1964), the relative sensitivity (the relative probability of an incident photon evoking a quantum bump) could be directly computed from such measurements. For the cell in Fig. 5, the light sensitivity of the R lobe was 940 times higher than that of the A lobe. In three other cells the ratios in light sensitivity between the R and A lobes were 510, 200, and 87.

The true difference in sensitivity between lobes should be higher than what we have measured since responses produced by illumination of the A lobe are at least in part due to light scattered into the R lobe by the optical system and by the cell itself. We investigated scattering by placing the spot so that it did not fall on the photoreceptor; it was placed the same distance from the R lobe as previously used to stimulate the A lobe but in the opposite direction. Light scattered from the spot was measured as an increase in quantum bump rate. The rates measured in this way were of the same order as those seen when directly illuminating the A lobe with the same spot. Therefore, light scatter into the R lobe contributed significantly to the apparent A-lobe sensitivity.

Light Adaptation Is Initiated at the R Lobe

To determine whether light adaptation is initiated primarily in the R lobe, an adapting spot was used to stimulate either the A lobe or the R lobe selectively (see Fig. 6). Subsequent changes in sensitivity were measured using a dim test spot incident on the A lobe. Reduction in quantum bump amplitude serves as a measure of the extent of light adaptation (Dodge et al., 1968). After an adapting spot was focused onto the R lobe, there was a much larger reduction of quantum bump amplitude (Fig. 6c) than after placing it on the A lobe (Fig. 6b). Thus, like excitation, light adaptation is initiated primarily in the R lobe.

DISCUSSION

As illustrated in Figs. 1, 3, and 4, *Limulus* ventral photoreceptors are subdivided into anatomically distinct lobes. In living preparations these lobes are obvious

only after removing the glia that surround the photoreceptors and this helps to explain why the lobes were not previously recognized. The lobes seen in denuded cells are not an artifact created by the denuding procedure because they can be readily identified in sections of intact ventral photoreceptors (Calman and Chamberlain, 1982).

We have shown by scanning electron microscopy that only R lobes have microvilli on their outer surface. This has been confirmed by Calman and Chamberlain (1982), who have identified microvilli on the outer surface of R lobes of ventral photoreceptors fixed *in vivo* for transmission electron microscopy. They further showed that all microvilli are restricted to the R-lobe

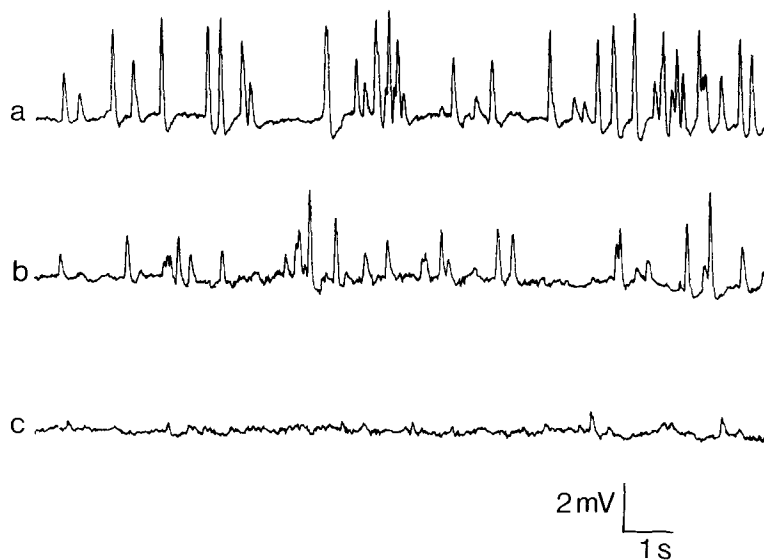


FIGURE 6. Effect of position of an adapting spot on subsequent sensitivity to light. (a) Dark-adapted response to test stimulus spot on A-lobe. (b) Response to same test stimulus after adapting light was placed on A-lobe. There was little adaptation. (c) Response to same test stimulus after adapting spot was placed on R-lobe. The greatly reduced quantum bump amplitude indicates that substantial light adaptation had occurred.

surface and to deep invaginations of the R-lobe. These invaginations may begin as the dimples seen on the R-lobes (Fig. 3*b*).

Two features of the microvilli as seen in the SEM are unexpected: first, the microvilli appear to be loosely packed, as opposed to the tight packing typical of invertebrate rhabdom and of ventral photoreceptors before denuding (Calman and Chamberlain, 1982); second, the microvilli are sometimes gnarled and fused. Structures of this kind have not been seen in the transmission electron microscope and may be artifacts caused by the denuding or fixation processes. In a previous study (Waterman and Pooley, 1980) of crustacean photoreceptors by scanning electron microscopy, microvilli retained an organized packing.

The R and A lobes are physiologically different. The R lobe is at least two to three orders of magnitude more sensitive to light than the A lobe. Microvilli are restricted to the surface of the R lobe and its infoldings (Calman and Chamberlain, 1982). Since the presence of microvilli greatly increases the membrane area, higher sensitivity of the R lobe to light is expected, even if rhodopsin is uniformly distributed within the cell membrane. Recent immunological evidence suggests that rhodopsin is present through both the rhabdomeric and nonrhabdomeric membrane of squid photoreceptors (Wong et al., 1982). Assuming that the A lobe in *Limulus* does contain a small amount of rhodopsin, it would be interesting to know whether this rhodopsin can initiate excitation or whether it is silent because it lacks some component associated with the microvillar structure that is necessary for transduction. Unfortunately, technical problems in our experiments make it unclear whether the low but finite sensitivity of the A lobe is real or whether its apparent sensitivity is an artifact caused by light scattered into the R lobe.

The initiation of light adaptation also occurs primarily in the R lobe (Fig. 6). Since light adaptation in *Limulus* appears to be mediated by a rise in intracellular free Ca^{2+} (Lisman and Brown, 1972, 1975) and since Ca^{2+} cannot readily diffuse through cytoplasm (Fein and Lisman, 1975), a reasonable expectation would be that the light-induced rise in Ca^{2+} would be largest in the R lobes. Harary and Brown (1981) have shown that the rise in Ca^{2+} produced by uniform illumination is spatially nonuniform within ventral photoreceptors, but it is not yet known whether the regions where the increase is large correspond to R lobes.

Similarly, we do not yet know how excitation processes spread from their site of initiation in the R lobe. Presumably a diffusible internal transmitter couples the isomerization of rhodopsin to the light-activated channels. From previous work (Fein and Charlton, 1975a), it is clear that the transmitter cannot spread uniformly around the cell, but it remains unclear how far excitation can spread and whether the light-activated channels are in the A lobe, the R lobe, or both. Experiments to examine these questions are underway.

The discovery that light sensitivity is concentrated in specialized lobes of ventral photoreceptors raises questions of interpretation about previous experiments which showed that spots of light positioned on opposite ends of a cell can locally excite (Fein and Charlton, 1975a) and adapt (Fein and Charlton, 1975b; Fein and Lisman, 1975) the cell. One interpretation of these experiments is that cells that showed independent effects at opposite ends (and not all cells did) had two R lobes, one positioned at each end. Another interpretation is that the spots were positioned on two electrically coupled cells, since what appears to be an isolated photoreceptor before denuding can sometimes be a pair of cells. This, however, cannot be the sole explanation because in some cases the presence of only a single cell was confirmed by dye injection (Fein and Charlton, 1975b). Finally, cells showing local effects may have had a R lobe running the length of the cell (Fig. 1d) which allowed distant parts of one R lobe to be locally illuminated.

The light responses of cells without their glia appear similar to the responses of normal cells. This implies that the glial cells are not intimately linked to phototransduction. Nevertheless, there may be important interactions between glia and photoreceptors. Glial cells buffer the ionic environment around photoreceptors (Coles and Tsacopoulos, 1981) and appear to provide long-term support of nerve cell function (Lasek and Tytell, 1981). Because their glial covering can be removed, the ventral photoreceptor preparation may be useful for further investigation of glia-photoreceptor interactions.

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