PHOTOELECTRIC SIGNALS GENERATED BY BOVINE ROD OUTER SEGMENT DISK MEMBRANES ATTACHED TO A LECITHIN BILAYER

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ABSTRACT Purified bovine rod outer segment disk membranes were attached to a lecithin bilayer membrane. After photoexcitation with a 500-nm flash delivered by a dye laser, a negative photovoltage was observed on the bilayer under normal ionic strengths (100 mM KCl), which had a rise phase of 1–3 ms at 20°C. The photoresponse was obviously due to bleaching of rhodopsin as it decreased for successive flashes of light. It originated most probably during the metarhodopsin-I metarhodopsin-II (meta-I-II) transition of rhodopsin because it was pH dependent at 2°C but not at 20°C. At 10 mM KCl, i.e., under hypotonic conditions, a positive photovoltage with slower kinetics than at high salt was observed. As the disk membranes were merely attached to the bilayer membrane, the photovoltage was apparently due to a light-induced transmembrane potential change in the disk membranes. Possible electrogenic mechanisms underlying the photosignal will be discussed.

INTRODUCTION

The generation of the receptor potential in the plasma membrane of the rod photoreceptor after light absorption is generally assumed to be mediated by an intracellular chemical transmitter (Baylor and Fuortes, 1970). Both, calcium ions and cyclic guanosine-3′, 5′-monophosphate (c-GMP) were suggested to act as transmitter substances (Hagins, 1972; Liebman and Pugh, 1979). The so-called Ca^{2+} hypothesis predicts a sudden permeability change of the disk membrane after photon absorption by rhodopsin. The c-GMP hypothesis suggests that light-induced enzyme activation, which leads ultimately to hydrolysis of c-GMP, triggers the receptor potential. Proof for both of these hypotheses does not exist at present. Moreover, recently there was an attempt to combine both hypotheses (Fatt, 1982).

A fast increase of the external Ca^{2+} concentration near the rod outer segment of intact photoreceptors after illumination was observed in two investigations (Gold and Korenbrot, 1980; Yoshikami et al., 1980). Although this finding indicates a fast light-induced Ca^{2+}-activity increase in the rod outer segments as predicted by the Ca^{2+} hypothesis, a fast Ca^{2+} release could not be demonstrated in the isolated photoreceptor membranes so far. Several attempts in this direction yielded only slow light-induced Ca^{2+} releases (Smith et al., 1977; Smith and Bauer, 1979; O'Brien, 1979).

Unfortunately, the transmembrane potentials correlated to ion movements through the disk membrane are not accessible to investigations with microelectrode methods due to the small size of the disks. Potential changes of disk membranes after illumination were estimated from the phase equilibria of appropriate hydrophobic paramagnetic ions (Cafiso and Hubbell, 1980*a*, *b*).

A different approach to investigate electric phenomena of photoreceptor membranes is to monitor electric signals from artificial lipid bilayers having photoreceptor membranes attached at one side (Fesenko and Lyubarskiy, 1977; Bamberg et al., 1980; Liu and Tien, 1982; Bauer et al., 1983). Related to these experiments, but less sensitive, are investigations of lipid impregnated Millipore filters (Lindau et al., 1980; Chapron, 1980) or collodion films (Drachev et al., 1981) containing photoreceptor membranes at one side. In response to bright light flashes, photosignals that are presumably due to the action of rhodopsin alone are derived from thin Teflon films coated with photoreceptor membranes after desintegration of the lipid structure with *n*-hexane (Trissl, 1979).

The signals obtained from these experiments, of course, depend on the complexity of the system under consideration. The simplest system involves purified osmotically intact disks or disk vesicles obtained by sonication of osmotically intact disks. Here, we report experiments carried out with these membrane preparations attached to one side of an artificial lecithin bilayer membrane.[1] A fast light-induced transient voltage is observed across the lecithin bilayer, which is apparently due to a transmembrane voltage of the disk membranes.

MATERIALS AND METHODS

Disk membranes were prepared by hypotonic treatment of purified fresh unfrozen bovine rod outer segments followed by flotation of the osmotically intact disks into a 5% Ficoll/water interface (Smith et al., 1975). All procedures were carried out at 4°C and in dim red light. Argon was used throughout to protect the disk membranes from oxidation. The floating band was washed free from Ficoll by pelleting at 80,000 *g* and 4°C for 6 min followed by resuspension in twice-destilled water and stored at 4°C until use.

Disk vesicles were prepared by sonication of a 1.75 ml sample containing 2.5 mg of rhodopsin buffered to pH 7.0. The sample was cooled to 4°C and then sonicated at resonance frequency for 20 s with the microtip probe of a sonifier (B12; Branson Sonic Power Co., Danbury, CT) at output meter reading of 60 W.

Lipid bilayer membranes were formed over a 1-mm hole in the diaphragm of a Teflon membrane cell following the technique of Müller and Rudin (Mueller et al., 1962). The experimental setup was recently described in detail (Bamberg et al., 1979; Fahr et al., 1981). A solution of 1% (wt/vol) L-α-diphytanoyl-lecithin (Avanti Polar Lipids, Birmingham, AL) dissolved in *n*-decane was used for membrane formation. A small amount of 0.025% (wt/vol) of octadecylamine was added to apply a positive electric charge to the lipid bilayer membrane (Danzsházy and Karvaly, 1976). The membrane was photoexcited with a 500-nm dye laser flash (model UV12/DL10; Molectron Corp., Sunnyvale, CA). The diameter of the light spot was <1 mm and illuminated only the lipid bilayer membrane. The energy of the laser flash was monitored after beam splitting with a millijoulemeter (model J3; Molectron Corp.).

The voltage between the two sides of the bilayer membrane was monitored using light-shielded Ag/AgCl electrodes connected to a high impedance amplifier (input resistance 10^{11} Ω). The voltage signal was either stored in a storage oscilloscope (7633; Tektronix, Inc., Beaverton, OR) or in digital form using a transient recorder (Biomation 8100, Gould Inc., Instruments Div., Santa Clara, CA).

Unless otherwise stated the buffers used for measurements at normal ionic strengths were either 5 mM Tris-maleate pH 7.0, 100 mM KCl, or 20 mM HEPES pH 7.0, 120 mM KCl; 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT). No difference in the photosignals between these two buffers was observed. At low ionic strengths, 0.5 mM Tris-maleate pH 7.0, 10 mM KCl was used.

RESULTS

When purified disk membranes were added to one side of a positively charged black lipid membrane, photoactivity began to develop and a voltage signal was recorded in response to a 500-nm laser flash. Under normal ionic strength a transient voltage signal was observed that was negative on the rhodopsin-containing side measured against the rhodopsin-free side. Due to the low diffusion coefficient of whole disks, the attachment of disks to the bilayer lipid membrane was a slow process, continuing for more than 1 h.

Similar experiments with bleached disk membranes yielded no photosignals. However, when disk membranes that were fully bleached and then regenerated by addition of excess 11-*cis*-retinal were used a negative photovoltage was again observed under these ionic conditions.

Much greater negative voltage signals were recorded when disk vesicles prepared by sonication of purified disks were used (Fig. 1). Under optimal conditions, amplitudes of several hundred microvolts were recorded after a flash bleaching a few percent of the rhodopsin contained in the attached disk vesicles. A small positive phase following the negative phase of the photosignal was often observed, which was sometimes hard to detect. For large photoresponses this positive phase was particularly prominent (Fig. 2). In response to successive 500-nm flashes of similar intensity, the amplitude of the voltage signal gradually decreased reaching a plateau value of ~10% of the initial value (Fig. 3). For this photostationary state, a proportionality between the amplitude of the photovoltage and the light intensity could be readily established. The photovoltage recorded in the photostationary state varied for different experiments, depending presumably on the amount of disk membranes attached to the lipid membrane.

At room temperature, no significant changes of the amplitude of the photovoltage were observed for different pH values between 6 and 8 pH (Fig. 4). However, at 2°C, higher photovoltages were recorded at 6 than at 8 pH. When the temperature was lowered from 20 to 2°C, the photovoltage decreased by a factor of ~2 at 6 pH and by a factor of ~6 at 8 pH.

To analyze the kinetics, up to five successive photores-

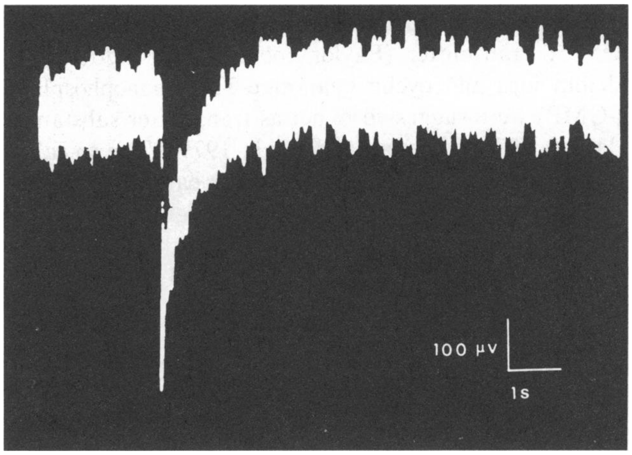

FIGURE 1 Photovoltage recorded at 20°C from a positively charged lecithin bilayer to which 180 µl of sonicated disk vesicles containing 0.27 mg of rhodopsin were added on one side 20 min before the measurement. The voltage is measured from the rhodopsin-containing side against the rhodopsin-free side of the lipid bilayer membrane. Energy of the 500 nm laser flash, 0.37 µJ; buffer, 20 mM HEPES, 120 mM KCl, 1 mM $MgCl_2$, 1 mM DTT, pH 8.5.

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[1] Short reports of these experiments were given previously (Bamberg et al., 1980; Bauer et al., 1983).

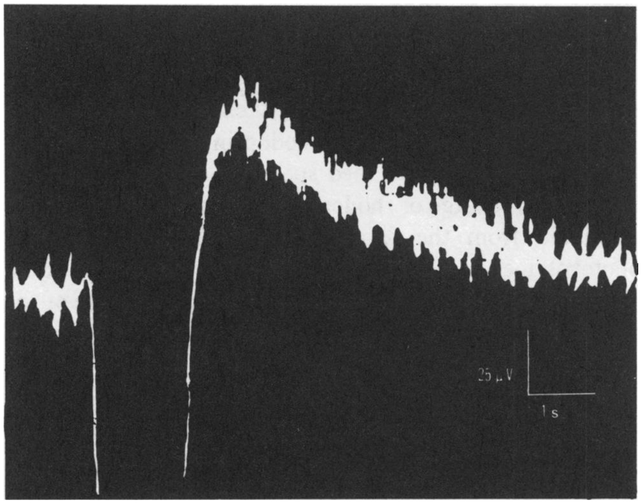

FIGURE 2 Photovoltage recorded from a lecithin bilayer to which sonicated disk vesicles were added on one side. The sonication was carried out in 67 mM KCl and 0.5 mM Tris-maleate pH 7.0. The amplitude of the negative peak is ~250 µV, the amplitude of the positive peak is 62 µV. For the polarity of the voltage see legend of Fig. 1. Light energy of the laser flash, 0.4 µJ; buffer, 0.5 mM Tris-maleate pH 7.0; temperature, 20°C.

ponses were superposed and averaged in digital form in a transient recorder and fitted with two exponentials yielding two relaxation times, τ_1 and τ_2, for the rise and the decay time. At 20°C and pH 7.0, the shorter relaxation time, τ_1, was 1–3 ms, somewhat shorter for vesicles than for whole disks. τ_1 was clearly temperature dependent with an activation energy of ~100 ± 10 kJ/mol. The relaxation time of the decay, τ_2, exhibited appreciable variation, depending on the pretreatment of the disks and the amount of bleached rhodopsin. Normally, it was 40–80 ms at room temperature.

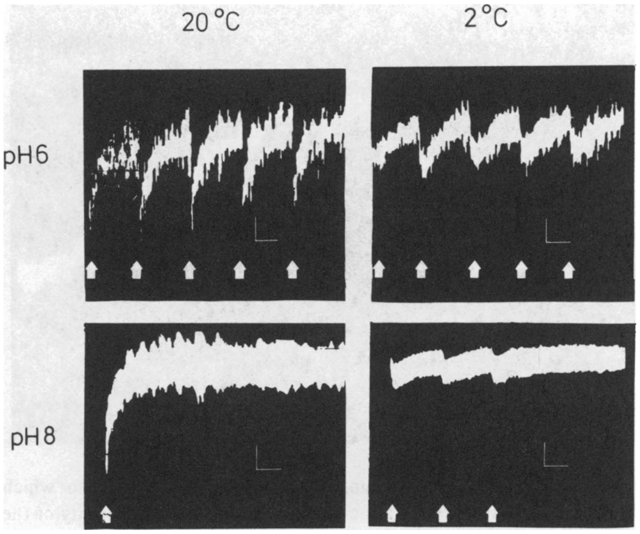

FIGURE 4 Photovoltage signals from a lecithin bilayer with disk vesicles attached on one side, recorded at different temperatures and pH values. The flash repetition rate was 2 s, the flash energy was ~0.25 µJ (500 nm). For the polarity of the voltage signal see legend of Fig. 1. At pH 8, the buffer was the same as in Fig. 1. At pH 6, HEPES was replaced by PIPES, the other ions being the same. The bars indicate 1 s and 100 µV.

All experiments reported so far were carried out in the presence of 100 mM KCl, i.e., under conditions where the disks were collapsed. The following experiment was carried out at lower ionic strength, using 10 mM KCl and 0.5 mM Tris-maleate at pH 7.0 in both compartments of the membrane cell. Under this condition osmotically intact disks are slightly swollen as found by Ficoll flotation and light scattering experiments (Bauer, P. J., and E. Mavrommati, manuscript in preparation). The photovoltage signal observed was then positive on the rhodopsin-containing side with respect to the rhodopsin-free side (Fig. 5) and the

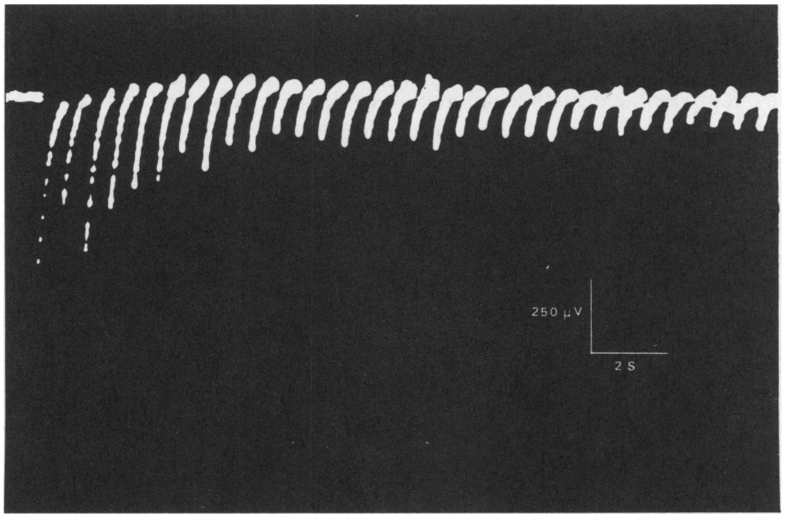

FIGURE 3 Photovoltage observed after excitation of the lipid bilayer membrane with repetitive flashes delivered at a rate of 2 flashes per second. For the polarity of the voltage signal see legend of Fig. 1. (The irregularity in the amplitudes was due to energy fluctuations of successive laser flashes in this experiment.)

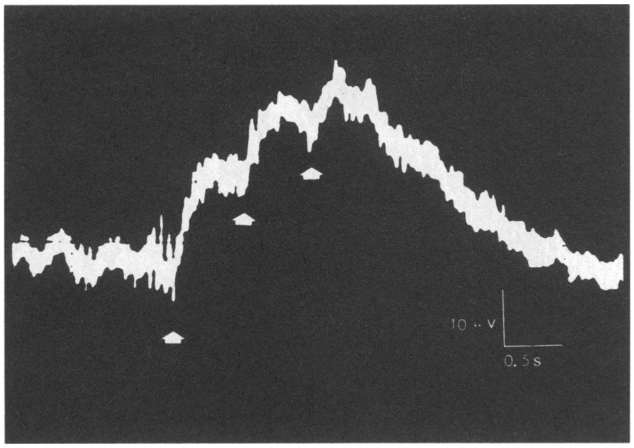

FIGURE 5 Photovoltage obtained from a lecithin bilayer to which osmotically intact disks are attached on one side. For the polarity of the voltage signal see legend of Fig. 1. Buffer, 0.5 mM Tris-maleate, pH 7, 10 mM KCl. Under this hypotonic condition the disks are osmotically slightly swollen (see text).

kinetics of both rise and decay phase were >10 times slower than the kinetics of the negative photoresponse.

DISCUSSION

Obviously, disk membranes have to interact with the positively charged lipid bilayer membrane to induce photosensitivity, because photosensitivity develops slowly after addition of the disk membranes. We ask whether this interaction consists in a mere attachment of the disk membranes to the bilayer or whether fusion, i.e., incorporation of disk membranes into the lipid bilayer membrane, occurs. Although a tight apposition of the disk membranes to the lipid bilayer membrane occurs, spontaneous fusion is unlikely because no large change in the bilayer membrane resistance was observed in these experiments after interaction with the disk membranes. Furthermore, an increase in the KCl concentration from 10 to 100 mM inverted the polarity of the photosignal. Although we have not yet done control experiments to rule out a possible influence of ionic strength, we suspect the effect is due to the change in osmotic pressure of the solution. We infer from these observations that intact disks and disk vesicles merely attach in a sandwichlike structure to the lecithin bilayer.

For this reason, the observed photovoltage is due to charge displacement across disk membranes that are closely attached to the bilayer. This process induces image charges on the rhodopsin-free side of the bilayer. Only disks that are attached to the bilayer membrane can contribute to the photovoltage since, otherwise, the electrolyte screens any electric field of the disks. Similar photoresponses have been reported for black lipid membranes in the presence of purple membrane sheets from *Halobacterium halobium* and for bacteriorhodopsin-containing vesicles, and the analysis of the photoresponse has been discussed (Bamberg et al., 1979; Fahr et al., 1981; Danzsázy and Karvaly, 1976; Rayfield, 1983).

A disadvantage of this experimental technique is that the amount of disk membranes attached to the bilayer, as well as the percentage of the rhodopsin bleached after one flash, cannot be determined spectroscopically. Nevertheless, the percentage of rhodopsin bleached per flash can be estimated from the decrease of the amplitude of the photovoltage in response to two weak flashes of equal intensities. This estimate implies that the amplitude of the photovoltage decreases proportionally to the fraction of rhodopsin bleached per flash.

The fraction of rhodopsin bleached can also be estimated theoretically from the energy of the laser flash since the laser flash is confined to the bilayer membrane. For this purpose, the extinction coefficient of rhodopsin at 500 nm of 40,000 l/mol/cm was converted into the cross section for photon capture of 1.53×10^{-16} cm^2. Assuming that the rhodopsin density in bovine disk membrane is not much different from the rhodopsin density measured for frog disks of 25,000 rhodopsin molecules per micron squared (Blasie et al., 1965), the probability for absorption of a 500-nm photon in a single disk is 0.076%. Thus, the fraction of rhodopsin bleached in the attached disks can be estimated without knowing the membrane area of the bilayer covered by disks as each disk will experience the same light intensity. Although several assumptions enter this calculation, we estimate from the measured light energy of the laser flash values for the fraction of rhodopsin bleached, which compare reasonably well with the values derived from the decrease in amplitude of the photosignal for two consecutive flashes (see above). Therefore, the observed fraction of bleached rhodopsin is in quantitative agreement with the assumption that only disks attached to the lipid bilayer membrane generate the photoresponse.

At 20°C, the kinetics of the rise phase are ~10 times faster and the decay kinetics 2–5 times slower than the kinetics of the metarhodopsin-I metarhodopsin-II (meta-I-II) transition (Lewis et al., 1981). The activation energy evaluated from the relaxation time of the rise phase is comparable with the activation energy of the meta-I-II transition (Lewis et al., 1981). This indicates that the photovoltage is triggered during the meta-I-II transition. Additional support for this view comes from the observation that at 2°C the photovoltage is pH dependent, being much smaller at pH 8 where meta-II is hardly produced than at pH 6, whereas at 20°C no pH dependence of the photovoltage was apparent in this pH range. Therefore, the meta-II formation and the generation of the photosignal have a similar pH dependence.

As discussed above, disk membranes are merely attached to the lecithin bilayer and, therefore, capacitively coupled to this supporting artificial membrane. For this reason, the observed photosignal is due to a light-induced transmembrane potential arising at the disk membrane after photon capture. Moreover, the negative polarity of the photosignal measured at normal ionic strength from the rhodopsin-containing side of the bilayer against the rhodopsin-free side means that this transmembrane potential is negative at the outside and positive at the inside of the disk. A similar conclusion was drawn from spin-label experiments (Cafiso and Hubbell, 1980*a*, *b*).

The basic question, of course, is: Which electrogenic mechanism of the disk membrane generates the observed photoresponse? From the experiments reported in this paper no definite answer can be given.

At first consideration, two electrogenic mechanisms occurring in disk membranes after light absorption should be considered as possible candidates for the photovoltage: (*a*) charge separation in rhodopsin and (*b*) proton binding occurring during the meta-I-II transition.

Charge separation in rhodopsin was postulated to generate the early receptor potential (Cone, 1967). More recent investigations indicate that interfacial charge separation occurs in the boundary regions of the disk membrane after photon capture (Cafiso and Hubbell, 1980*a*, *b*). Light-induced charge separation in rhodopsin constitutes a mechanism that is localized in rhodopsin itself. Because in the collapsed disk two rhodopsin-containing membranes are inversely opposed, the electric fields of these oppositely located rhodopsin molecules will almost compensate for each other. One should expect, however, that after osmotic swelling this compensating effect decreases. Therefore, the signal should become greater for partially swollen disks if this mechanism generates the observed photoresponse. In contrast to this prediction, a positive signal was observed under hypotonic conditions (Fig. 5). One may speculate that interaction of rhodopsin in the collapsed disks is a necessary precondition for the generation of the negative photosignal; there is, however, not much evidence in favor of such a hypothesis. Moreover, it appears unlikely that the same mechanism that evokes the early receptor potential is also the ionic mechanism underlying this photoresponse as under optimal conditions the photosignal can be observed after light flashes, which bleach only a few percent of rhodopsin, whereas it is a well-known characteristic of the early receptor potential that it can only be detected after unusually intense light flashes (Cone, 1967).

Another electrogenic mechanism mentioned above is light-induced protonation of rhodopsin, which is known to occur during the meta-I-II transition of rhodopsin (Matthews et al., 1963). Protonation of rhodopsin occurs in milliseconds after photon capture and may result in an inside positive transmembrane potential of the disk membranes (Cafiso and Hubbell, 1980*a*). In agreement with our experimental result one would expect that the observed photovoltage generated by protonation should decrease when the disks swell osmotically. If protonation evokes the negative photosignal observed at normal ionic strength, one has to assume that the positive signal, which was recorded at low osmolarity, is due to another ionic mechanism. It is possible that this photosignal is identical to the positive phase described in Fig. 2.

The negative photosignal may also be due to a light-induced cation permeability increase of disk membranes. As the disks are prepared under low ionic strength and then added to 100 mM KCl in the actual experiment, the ionic gradient points from the outside to the disk interior. For this reason, a potassium permeability increase of the disks would result in an inside positive potassium diffusion potential. At low ionic strength, i.e., at 10 mM KCl, the potassium gradient is ~10 times smaller and, therefore, the diffusion potential may be masked by the positive phase (Fig. 2).

Although we cannot decide at present which ionic mechanism generates the observed negative photoresponse, the latter mechanism appears most likely to us. Recently a light-induced permeability change of disk membranes to potassium ions was also suggested from spin-label experiments (Cafiso and Hubbell, 1981). More detailed investigations are necessary, however, to prove or disprove this hypothesis.

We cannot exclude the possibility that more than one ionic process contributes to the observed photoresponse. In particular, the origin of the small positive phase is not yet clear. Preliminary experiments indicate that it can be influenced by the proton carrier carbonylcyanide-*p*-trifluoromethoxyphenylhydrazone (FCCP). Furthermore, one might expect that disk membranes increase the ionic permeability at low osmotic pressure. On the other hand, it was observed that artifical lipid membranes under similar osmotic conditions show no change in conductivity.

In summary, we find under normal ionic conditions a light-induced inside positive transmembrane potential across the disk membranes that, under many conditions, is followed by a small voltage of opposite polarity. This result can be explained by assuming that the permeability of the disk membranes increases transiently after illumination, which supports recent spin-label experiments (Cafiso and Hubbell, 1981). However, we cannot rule out other mechanisms at present or the simultaneous contribution of several ionic mechanisms to the observed photosignal. Further experiments will be necessary to elucidate the electrogenic mechanism that generates the observed photovoltage.

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