Light-Activated Calcium Release from Sonicated Bovine Retinal Rod Outer Segment Disks†

H. Gilbert Smith,‡ Jr., Roger S. Fager,* and Burton J. Litman

ABSTRACT: Calcium trapped within sonicated and resealed bovine rod outer segment disks is released upon light exposure with a stoichiometry of 0.75 ± 0.05 calcium for each rhodopsin bleached. The amount of calcium liberated is proportional to the amount of bleaching in the range of 20 to 100% bleaching and is relatively insensitive to the internal trapped calcium concentration. These results are obtained using a flow system in which the disk membrane vesicles are adsorbed on glass particle supported by a filter. The external calcium is washed away and subsequent calcium release is monitored by collecting fractions of the effluent before, during, and after light exposure. Disks that are sonicated and allowed to reseal prior to incubation with ^{45}Ca show no change in calcium efflux upon bleaching. The light-activated calcium release is also eliminated if disks sonicated in the presence of ^{45}Ca are treated with a calcium ionophore prior to bleaching. These results demonstrate that the light-released calcium comes from within the disks and not from the external disk surface. Lowering the temperature to 3–4 °C surpresses the light-stimulated release, implicating a transition after the formation of metarhodopsin I in the transport process. These results suggest a model for the disk in which each bleached rhodopsin functions as a "one-shot carrier" to transport a single calcium ion across the membrane.

The initial event in scotopic vision is the absorption of light by the photopigment rhodopsin which is located primarily in the disk membranes of the rod cells of the retina. Upon absorption of a photon of light, the retinal chromophore of rhodopsin isomerizes from the 11-cis to the all-trans configuration (Hubbard and Kropf, 1958; Wald, 1968). This isomerization initiates processes that lead to a reduced sodium current through the photoreceptor plasma membrane and consequently to a hyperpolarization of that membrane (Hagins, 1972; Tomita, 1972; Sillman et al., 1969; Yoshikami and Hagins, 1973). Since the disk membranes are separate from the plasma membrane (Cohen, 1972), a signal must be transmitted from the disks to the plasma membrane to trigger the hyperpolarization. Hagins and Yoshikami have proposed that this signal is carried by calcium ions which are released from within the disks when rhodopsin is bleached and which subsequently diffuse to the plasma membrane to block sodium channels (Hagins, 1972; Hagins and Yoshikami, 1974; Yoshikami and Hagins, 1973).

The validity of this model has been supported by electrophysiological studies in which calcium mimics the effect of light by reducing the sodium current through the plasma membrane (Hagins and Yoshikami, 1974; Yoshikami and Hagins, 1973). Studies of the osmotic swelling and shrinking of isolated rod outer segments also indicate that calcium reduces the sodium permeability of the plasma membrane (Bownds and Brodie, 1975; Korenbrot and Cone, 1972; Wormington and Cone, 1975). Additional information is provided by measurements of the light-stimulated release of radioactive calcium that has been trapped within disks by sonication (Mason et al., 1974); these measurements suggest that each bleached rhodopsin releases a single calcium ion. Further confirmation has been sought in attempts to look directly for a light-stimulated release of calcium from disk membranes. Hendricks et al. (1974), Liebman (1974), and Szuts and Cone (Poo and Cone, 1973; Szuts and Cone, 1974) used atomic absorption measurements to study the light-stimulated release of calcium. Szuts and Cone reported that some of their experiments showed 10 to 1000 calcium ions released for each rhodopsin bleached in contrast to a significantly smaller release observed by Liebman and by Hendricks et al.

The accumulation and release of calcium from intact photoreceptor membranes after incubation with radioactive calcium have also been examined, although with conflicting results. Hemminki (1975a,b) has reported that the binding of calcium to photoreceptor membranes is reduced upon light exposure by about one calcium for every three rhodopsin molecules bleached; however, other workers have found light to have no effect on the accumulation of calcium (Bownds et al., 1971; Neufeld et al., 1972; Weller et al., 1975). It is not clear in these studies whether the calcium taken up is bound to the membrane surface or actively pumped into the disks. Sorbi and Cavaggioni (1975) and Bownds et al. (1971) find light to have a negligible effect on the rate of loss of previously accumulated calcium. Hemminki (1975a) does see a difference in the rate of loss from dark and light samples that corresponds to about one calcium released for every six rhodopsins bleached, and Weller et al. (1975) see a smaller release of about one calcium for every 100 rhodopsins bleached. Hemminki's (1975b) experiments suggest that the light-released calcium comes from binding sites on the membrane, in contrast to Weller et al. (1975) who argue that the effect of light is on the permeability of the membrane.

We have examined the light-activated release of calcium from disk membranes in a flow apparatus that allows a direct measurement of the released calcium on a shorter time scale than possible with other published methods. The flow system eliminates the uncertainties created by passive leakage in a

† From the Department of Biochemistry (H.G.S. and B.J.L.) and the Department of Physiology (R.S.F.), School of Medicine, University of Virginia, Charlottesville, Virginia 22901. *Received July 29, 1976.* This research was supported by the National Institutes of Health (Grant EY00548 to B.J.L. and Grant EY01505 to R.S.F.) and by the National Science Foundation (Grant GB 41313 to B.J.L.). A preliminary report of this research was given at the 1976 meeting of the Americal Physiological Society (*The Physiologist 19*, 371).

‡ Supported by a NIH Training Grant.

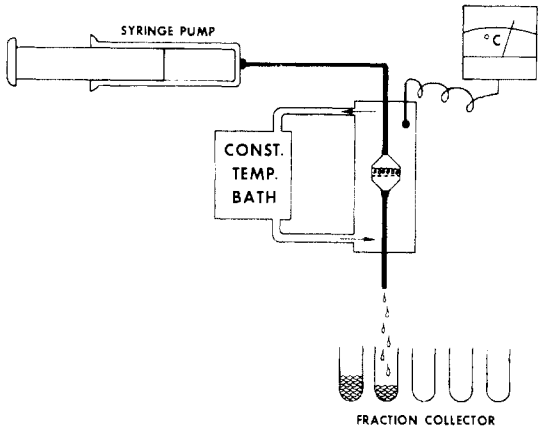

FIGURE 1: Flow apparatus used in calcium-release experiments.

static system. Our data indicate that upon light exposure there is a stoichiometric release of calcium from disk membranes by a "one-shot carrier" mechanism, that is, a mechanism in which each bleached rhodopsin releases a single calcium from within the disk. The temperature dependence of this release implicates a transition in the bleaching process after the formation of metarhodopsin I.

Materials and Methods

General Procedures. Unless otherwise specified, the detergent and buffer solutions used were at pH 7.4 and the procedures were carried out under dim red light (15-W bulb, Wratten safelight no. 1, Eastman Kodak). The pH of the imidazole buffer is adjusted with HCl. Rhodopsin concentrations were determined from the change in absorbance at 500 nm (ΔA_{500}) upon bleaching a solubilized sample in the presence of hydroxylamine. The molar extinction coefficient used was 40 000; the molecular weight was 40 000.

Disk Preparation. Disks were prepared from frozen bovine retinas (Hormel) by a modification of the method of Smith et al. (1975). The water wash was omitted and instead the pelleted rod outer segments were suspended directly in 5% Ficoll (Sigma). After 2 h in the refrigerator, the rods had burst and the disks could be harvested from the 5% Ficoll–water interface after centrifugation at 80 900g for 2 h. The resulting disk suspension was stored under argon in a freezer. Solubilized aliquots of this material routinely had A_{278}/A_{498} ratios of 2.1.

Calcium Trapping. Calcium was trapped by sonicating a suspension of disks containing about 1 mg of rhodopsin in a 1-ml final volume of 0.1 M imidazole buffer that contained 50 μCi of ^{45}Ca and 15 μmol of calcium chloride. The sonicated material was left in the refrigerator overnight to allow for resealing of the membranes (Mason and Lee, 1973). In some experiments, other levels of calcium were used as described in the text and, in some cases, a final volume of 0.5 mL was used. The sonication was done in a small glass vial using the microprobe of a Branson Sonifier (Model 350W) with an output of about 75 W. During sonication, the sample was kept under argon and was cooled in an ice–water bath. The sonication time was 15–30 s.

Calcium-Release Experiments. The apparatus used consists of a syringe pump (Harvard Apparatus, Inc., Model 904), a modified 13-mm Millipore filter holder, and an LKB Mini-Rak fraction collector (see Figure 1). The filter holder was mounted in a piece of Plexiglas tubing that was connected to a thermostated water supply. Temperature in the jacket was monitored with a YSI thermistor probe connected to a Model 42 Telethermometer. The opaque top of the Millipore filter holder was replaced with one made of clear Plexiglas so that samples in the filter holder could be bleached. A 0.4-μm pore diameter Nucleopore filter was mounted in the holder and layered with a bed of Corning CPG-550 controlled pore glass (5–10-μm particle diameter). The sonicated disks adhered to these glass particles making it possible to wash the external calcium away with a continuous stream of buffer. The light-stimulated calcium release was then measured by monitoring the radioactivity of the effluent before, during, and after bleaching.

In a typical experiment, 0.5 mL of a 20 mg/mL suspension of glass particles in buffer was injected into the cell to give a bed volume of about 0.1 mL and then washed with about 2 mL of buffer to pack the bed. A 0.1-mL aliquot of the sonicated disks (about 0.1 mg of rhodopsin) was then loaded into the filter holder and washed with 50 mL of 0.1 M imidazole buffer at a flow rate of 2 mL/min to remove external calcium. After this prewash, 0.8-mL fractions were collected at a flow rate of 1 mL/min. If buffers other than 0.1 M imidazole were used, they were introduced at this time. After about 15 fractions had been collected, the sample was bleached using light from a microscope lamp for the full bleaches or room light for the partial bleaches. Fraction collection continued without interruption to a total of about 35 fractions. Finally, the membranes were eluted from the glass particles by washing with 5% CTAB,[1] 10 mM EGTA, 0.2 M NH_2OH in 0.1 M imidazole buffer. These samples were counted to determine the amount of calcium remaining with the sonicated disks after bleaching. Fractions were collected directly into Demuth Glass Co. Petite scintillation vials and were counted in a Beckman, Model LS-230, liquid scintillation counter using 5 mL of a scintillation cocktail composed of 5.5 g of 2,5-diphenyloxazole, 0.125 g of Me_2POPOP, 333 mL of Triton X-100 (Rhom and Haas), and 666 mL of toluene. The specific activity was determined by counting a small aliquot of the original sonicated disk suspension. The external standard method was used to make corrections for counting efficiency.

In some experiments, calcium ionophores were introduced into the flow system. One milliliter of 10 μM X-537A (Hoffmann-LaRoche) or 0.4 μM A23187 (Eli Lilly) in 0.1 M imidazole was injected into the system instead of bleaching to measure the amount of calcium trapped within the disks. Experiments were also done in which the ionophores were introduced before or after bleaching. These experiments showed that rhodopsin and the ionophore draw from a common calcium pool. Suspensions of the ionophores were prepared by injecting 1.0 μL of an ethanolic solution of the ionophore into 1 mL of 0.1 M imidazole buffer.

TNBS Labeling of Lipids in Sonicated Disks. The lipid asymmetry of the sonicated disk membranes was measured using the nonmembrane penetrating reagent, TNBS, in a manner analogous to the previously employed for phospholipid vesicles (Litman, 1974). This reagent reacts with primary amino groups under basic conditions and the reaction can be stopped by acidification. The ratio of external to total primary amino groups was determined by comparing the amount of labeling in an intact sample with the labeling of a sample solubilized in the detergent, Triton X-100.

[1] Abbreviations used are: CTAB, cetyltrimethylammonium bromide; TNBS, 2,4,6-trinitrobenzenesulfonic acid; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; POPOP, 1,4-bis[2-(5-phenyloxazolyl)]benzene; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

Disks were suspended in a 2-mL final volume of 15 mM $CaCl_2$, 0.1 M imidazole buffer to give a final rhodopsin concentration of 2.0 mg/mL (based upon ΔA_{500}). One milliliter of this suspension was then sonicated as described for the calcium-release experiments. The intact and sonicated samples were each diluted with 1.2 mL of 0.8 M $NaHCO_3$ buffer (pH 8.5) and labeling was begun by the addition of 0.3 mL of 1.5% TNBS. At various times, 0.6-mL aliquots of the suspension were removed and added to 15 mL of cold 0.2 M sodium acetate buffer (pH 5.0) to stop the reaction. The labeled membranes were collected by centrifugation at 20 000 rpm in a Sorvall SS-34 rotor. Unreacted TNBS was removed by washing the pellet twice more with the sodium acetate buffer. The pellet from the second wash was resuspended by homogenization in 2 mL of water and divided into six 0.3-mL aliquots, each of which was then extracted with 1.2 mL of 2:1 chloroform–methanol (v/v). The extracted lipids were dried under a stream of dry nitrogen and the samples were then dissolved in 0.8 mL of 0.2 M sodium bicarbonate buffer (pH 8.5) that contained 0.4% Triton X-100. Three of these solubilized samples were immediately acidified with 0.4 mL of 0.4% Triton X-100 in 1.5 M HCl and the absorbance was read at 410 nm to determine the amount of labeled lipid on the external surface. Each of the three remaining samples was reacted with an additional 20 μL of 1.5% TNBS to determine the total amount of primary amino groups in the sample. After a 30-min reaction time, these samples were acidified and the absorbance at 410 nm was measured. All absorbances were read against a reagent blank. The ratio of the absorbance of the externally labeled samples to that of the totally labeled samples gave the percentage of lipid primary amino groups (phosphatidylethanolamine and phosphatidylserine) on the external surface of the disks.

To determine if the disk membrane is permeable to TNBS under the conditions of these labeling experiments, we also sonicated disks in the presence of 0.3 M arginine. These sonicated disks were loaded into a 25-mm filter holder that contained 0.1 g of the 5–10-μm glass particles supported by a 0.4-μm pore diameter nucleopore filter. The external arginine was washed away with 9 mL of 0.8 M sodium bicarbonate buffer (pH 8.5) and TNBS was then added to the system. TNBS leakage would result in labeling of trapped arginine. The samples were extracted with 2:1 chloroform–methanol and the aqueous layer of this extraction was analyzed for labeled arginine.

Regeneration of Sonicated Disks after Binding to Glass Particles. These experiments were conducted to determine if significant rhodopsin denaturation takes place during the calcium-release procedures. Disks were sonicated as described above, but without the radioactive calcium. An aliquot of these sonicated disks was loaded into the filter holder which contained glass particles, and treated as in the normal release experiment, except that the membranes were not eluted with detergent. The filter, glass particles, and adhering bleached membranes were added to 0.5 mL of 0.1 M imidazole buffer, 0.5 μL of 25 mM ethanolic 11-*cis*-retinal was added, and the sample was incubated for 1 h at 37 °C. A sample of sonicated disks suspended directly in buffer was similarly regenerated as was a sample added to a suspension of glass particles (without being loaded on the filter and washed). Dark samples were run as controls for the recovery of material. After incubation, the membranes were solubilized with 0.5 mL of 5% CTAB, 0.2 M NH_2OH, 0.1 M imidazole, and the glass particles were removed by filtering with a 0.8-μm Nucleopore filter. The percentage regeneration was computed by comparing the ΔA_{500} of a regenerated sample with that of an appropriate dark sample.

Samples were also regenerated in a similar manner in the presence of detergents. One-half milliliter of 2% digitonin, 0.1 M imidazole was added to each sample before the 11-*cis*-retinal and the CTAB solubilization was omitted. 0.02 mL of 2.5 M NH_2OH (pH 7.1) was added before measuring the ΔA_{500}. Regeneration in the presence of octyl glucoside was done similarly, except that the 11-*cis*-retinal was added to the 75 mM detergent solution before being added to the membrane suspension and the samples were incubated for 2 h at room temperature. The octyl glucoside was prepared as described previously (Stubbs et al., 1976).

Preparation of Sonicated Disks from Regenerated Membranes. A 0.5-mL suspension of disks in 0.1 M imidazole buffer with a rhodopsin concentration of 2 mg/mL was fully bleached. Then, 2.5 μl of 25 mM ethanolic 11-*cis*-retinal was added and the sample was incubated at room temperature for 2 h. A 0.25 mL aliquot of this regenerated sample was sonicated for 20 s after addition of 0.25 mL of imidazole buffer that contained 25 μCi of ^{45}Ca and 7.5 μmol of $CaCl_2$. After being left in the refrigerator overnight to reseal, 0.1 mL of the sonicated suspension was loaded onto the glass particles in the filter holder and a normal calcium-release experiment was conducted.

Results

Binding of Sonicated Disks to Porous Glass Particles. The amount of material bound to the glass particles, after washing with buffer as in the normal experiment, was measured by eluting an unbleached sample from the filter with a 5% solution of the detergent CTAB in 0.2 M NH_2OH, 0.1 M imidazole and spectroscopically determining the rhodopsin recovery relative to the original loading. The measured recoveries are 81 to 87% at various pH between 6.2 and 7.4. If 37–74-μm glass particles were used, instead of the usual 5–10-μm particles, then only a 50–55% recovery was obtained and if no glass particles were used only a 10% recovery was observed. The large external surface area of the small glass particles is thus seen to be important for retention of the sonicated disks. Recovery after bleaching was measured by a Lowry (Lowry et al., 1951) determination of the protein adhering to the glass particles and filter at the end of a typical experiment (before detergent elution). These measurements indicate an 88% recovery and demonstrate that there is no loss of material during bleaching. An 85% binding efficiency is assumed for the calculations in this paper.

Lipid Structure of Sonicated Disks Compared to Intact Disks. We have used the nonmembrane penetrating reagent TNBS to study the orientation of the lipids in intact and sonicated disks. In both cases, 100% of the lipid primary amino groups react with the reagent within 30 min. In separate experiments, it was found that arginine trapped within disks by sonication was not labeled by externally added TNBS. This indicates that TNBS does not leak into the disks and that the TNBS-labeled lipids are, in fact, on the outer-membrane surface. These results indicate that there is a marked lipid asymmetry across the membranes of both intact and sonicated disks with all of the phosphatidylethanolamine and phosphatidylserine being in the outer leaflet of the membrane bilayer. By this measure, there is not a gross reorganization of the membrane upon sonication.

Light-Activated Calcium Release. Data from typical calcium-release experiments are shown in Figure 2. Upon light exposure, there is a release of calcium into the effluent which

TABLE I: Relationship between Bleaching and Calcium Release.

Bleaching Time (s)	% Rhodopsin Bleached	Rel % Bleached	Rel Peak Ht[a]	Rel Peak Area[a]	Calcium Release[b] / Rhodopsin Bleached
60	57	1.00	1.00	1.00	1.00
40	43	0.75	0.80 ± 0.11	0.82 ± 0.16	1.09 ± 0.21
20	24	0.42	0.42 ± 0.10	0.38 ± 0.15	0.91 ± 0.35

[a] Mean values ± standard deviation from three experiments of the type shown in Figure 3. [b] Calculated from relative % rhodopsin bleached and relative peak areas. Normalized to 60-s results.

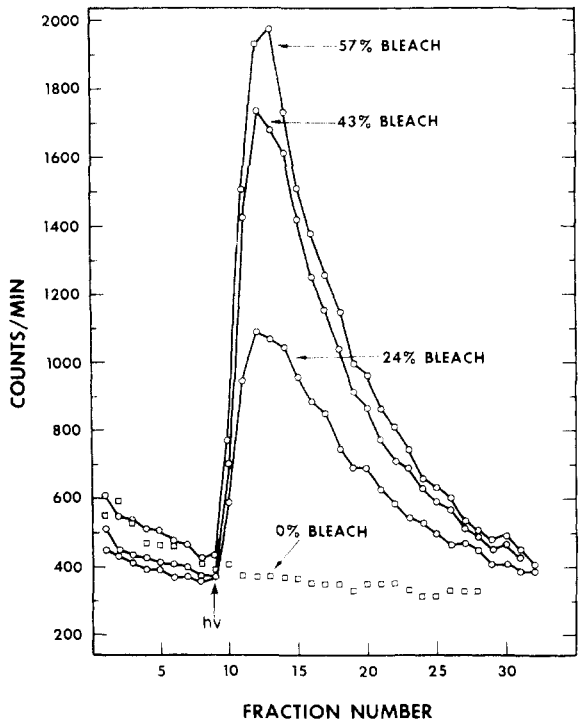

FIGURE 2: Light-activated calcium release. 0.8-mL fractions were collected at a flow rate of 1 mL/min after a 50-mL prewash, as described under Materials and Methods. The percent bleaching was determined on separate samples by eluting the membranes from the filter with detergent after bleaching, and measuring the ΔA_{500}. The samples were bleached with room light beginning with the fraction marked $h\nu$ (○) or not bleached at all as a control (□). Bleaching times were 20 s for 24% bleaching, 40 s for 43%, and 60 s for 57%. The sample activities are all 7.0×10^6 cpm/μmol of Ca.

is characterized by a rapid rise followed by a slower decay. The area under the curve represents the total amount of calcium released and is proportional to the amount of bleaching, as shown in Table I. The peak heights show this same proportionality, indicating that the rate constants for the release are the same at all levels of bleaching. In total-bleaching experiments, the stoichiometry of the release is determined to be 0.74 ± 0.05 calcium molecules released for each rhodopsin (mean ± standard deviation from eight experiments). Further evidence that the release is stoichiometric with the amount of rhodopsin bleached is provided by the data of Figure 3; this shows calcium to be released by both an initial 43% bleach and a subsequent total bleach of the same sample. We have been unable to obtain similar release from regenerated material. Bleached disks that had been 80% regenerated with 11-*cis*-retinal before sonication gave only about 10% of the usual release. A control experiment in which the disks were not bleached before the addition of 11-*cis*-retinal showed about 35% of the usual release.

Externally-Bound Calcium. To determine if the light-activated release includes calcium bound to the external surface of the membranes, we sonicated disks in the absence of ^{45}Ca. After the membranes had been left overnight to reseal, we added the same amount of ^{45}Ca as in the normal sonication procedure. This ^{45}Ca should exchange with any externally bound calcium and experiments with this material should measure only release from the external surface of the sonicated disks. In fact, no light-stimulated release is seen, after incubation with the external calcium for 10 min, 3 h, or 3 days. This is in agreement with similar experiments conducted by others (Bownds et al., 1971; Sorbi and Cavaggioni, 1975). These results indicate that the released calcium comes from a site that is not loaded from the external surface of the membrane. Similar experiments in which intact disks were incubated with ^{45}Ca also give no release upon light exposure. Further evidence that the released calcium comes from within the sonicated disks, and not from the external surface, was obtained in experiments with calcium ionophores. If 1 mL of 10 μM X-537A or 0.4 μM A 23187 in the imidazole buffer was injected into the flow system in place of the usual bleaching, there was a release of calcium larger than that seen in the normal light release. Subsequent bleaching showed no light-releasable calcium remaining in the ionophore-treated disks.

Effect of Internal Calcium Concentration. The concentration of calcium within the sonicated disks was varied by altering the amounts of calcium used during sonication. In total bleaching experiments, disks sonicated in 7.5, 15, 30, 60, or 120 mM calcium gave, respectively, releases of 0.25, 0.74, 0.55, 0.55, or 0.42 calcium per rhodopsin. There was noticeable aggregation in the sample sonicated with 120 mM calcium. Although the exact concentrations of internal calcium are not known because of the presence of endogenous calcium and losses by passive leakage, the experiments do show that the amount of calcium released upon total bleaching is relatively insensitive to the internal calcium concentration if this concentration is at least 15 mM. Experiments in which the disks are treated with calcium ionophores also give information on the amount of internal calcium. The amount of ionophore-releasable calcium in disks sonicated with 30 mM calcium is 1.6 times that found in disks sonicated with 15 mM calcium. The fact that the ionophore treatment eliminates the light-activated release indicates that the ionophore and rhodopsin draw from the same calcium pool. This is further substantiated by experiments which show less ionophore-induced calcium release when the ionophore is added after bleaching than when the ionophore is added before bleaching. With both 15 and 30 mM samples, this reduction can be accounted for by the amount of calcium released by bleaching.

Calcium Release from Sonicated Rod Outer Segments. To determine if a factor necessary for amplified calcium release is lost in the disk preparation, we did a series of experiments using sonicated rod outer segments, instead of isolated disks.

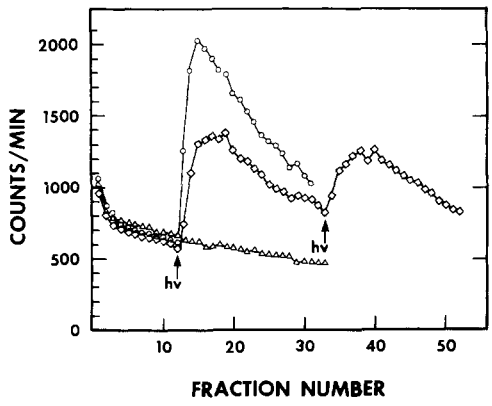

FIGURE 3: Release of calcium during sequential partial bleaches. The sample was initially treated in the same way as the 43% bleach of Figure 3 then, after most of the calcium released by this 40-s bleach had been eluted, the light was again turned on to bleach the remaining rhodopsin (◇). Experiments in which the samples are fully bleached (○) or not bleached (△) are shown for comparison. The sample activities are 1.56×10^7 cpm/μmol of Ca.

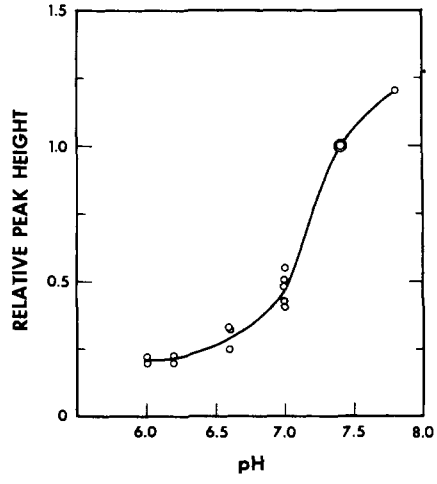

FIGURE 4: Dependence of calcium release upon pH. The peak heights above baseline for experiments at various pH are normalized to the peak height for a pH 7.4 experiment that used the same stock suspension of sonicated disks.

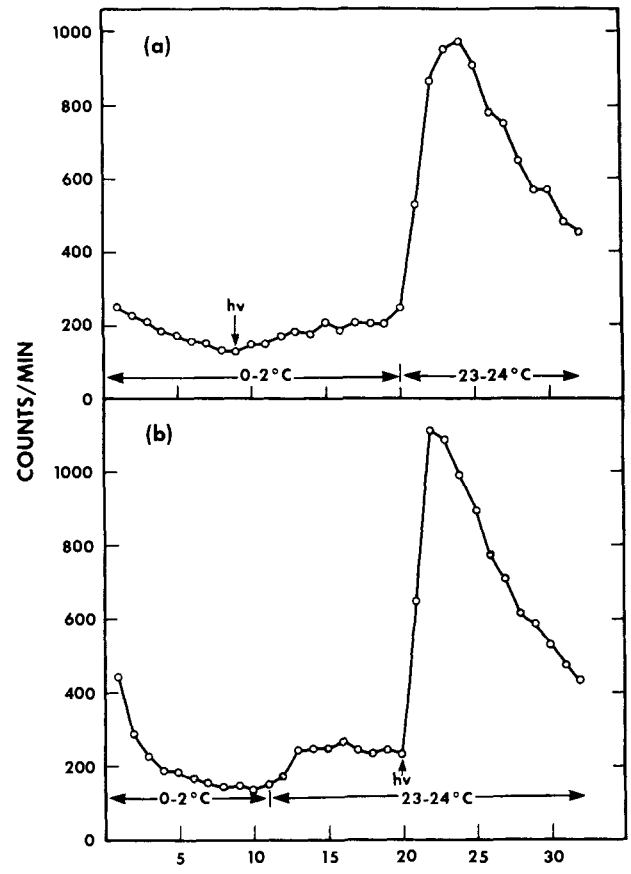

FIGURE 5: Effect of temperature on calcium release. After the prewash with 50 mL of 0.1 M imidazole (pH 7.4) at 23–24 °C, the temperature was lowered to 0–2 °C and fractions were collected in the normal manner. In a the sample was bleached during collection of the fraction marked *hν*, and the temperature was subsequently raised to 23–24 °C as indicated; b represents a similar experiment in which the temperature was raised before bleaching. The temperature was controlled by switching the supply of water to the jacket between two thermostated sources. The temperatures recorded are those measured in the jacket surrounding the filter holder. The sample activities are 5.29×10^6 cpm/μmol of Ca.

The amount of calcium released upon light exposure was about the same as for the disks but a much higher background was observed and about 100 times more counts remained after bleaching than remain when sonicated disks are used. Thus, although the rods or impurities in the rod preparation bind more calcium than do the disks, the amount of calcium released by each bleached rhodopsin is about the same as with the purified disks.

Effect of Buffers on Calcium Release. Most of the experiments used 0.1 M imidazole buffer (pH 7.4). If Tris buffer of the same pH was used, only about one-half as large a release was seen as with the imidazole. An even smaller release was seen if Pipes buffer was used and the release was further reduced if the buffer contained 10 mM EGTA. Addition of 5 mM KCl, 1.5 mM $CaCl_2$, or 0.15 M NaCl to the imidazole buffer had no effect on the release.

pH Studies. The amount of calcium released upon light exposure increases with increasing pH over the pH range from pH 6.0 to 7.8, as shown in Figure 4. The initial wash to remove external calcium was with pH 7.4 imidazole buffer in each case and the buffer was changed to the pH indicated when fraction collection began. The peak heights are normalized to the pH 7.4 results.

Temperature Studies. When the filter holder was thermostated at 0–2 °C, no release was seen upon bleaching; however, if the temperature was subsequently raised to 23–24 °C in the dark, there was a release comparable to that seen in the normal bleaching experiments. There was only a small shift in the baseline when the temperature was raised in a control experiment that omitted the low-temperature bleach. This sample gave a normal release when subsequently bleached at 23–24 °C. These results are shown in Figure 5.

The 500-nm absorption maximum of sonicated disks shifts to 480 nm upon bleaching at 2.5 °C, which indicates that the decay of rhodopsin intermediates proceeds only to metarhodopsin I at this temperature. The absorption spectra were measured in the presence of hydroxylamine using a bleached room-temperature sample as a scattering blank.

Regenerability of Material Bound to Glass Particles. Sonicated disks bound to the glass particles, as in the release experiments, are only slightly less regenerable than similar material in the absence of the glass particles (Table II). In the presence of detergent, material treated as in the release experiments and material bleached in the absence of glass particles are equally regenerable.

TABLE II: Effect of Disk Treatment upon Regeneration.

Regeneration Conditions	Regenerability Treatment I[a] (%)	Treatment II[b] (%)	Treatment III[c] (%)
Buffer[e] (n = 3)	79 ± 1[d]	86 ± 5[d]	86 ± 3[d]
Buffer + 1% digitonin (n = 1)	70	71	66
Buffer + 38 mM octyl-glucoside (n = 1)	48	48	48

[a] Treatment I: Material from normal experiment. Values are adjusted for 85% recovery after buffer washes. [b] Treatment II: Material bleached while in suspension with glass beads. [c] Treatment III: Material bleached while in suspension without glass beads. [d] Mean ± standard deviation. [e] Buffer: 0.1 M imidazole, pH 7.4.

Discussion

The results presented in this paper demonstrate that light can trigger the release of calcium from sonicated disk membranes and support calcium as the signal carrier in the model of Hagins and Yoshikami (Hagins, 1972; Hagins and Yoshikami, 1974; Yoshikami and Hagins, 1973). The TNBS-labeling studies suggest that the sonicated membranes have the same sidedness as the disks themselves and are not inverted during the sonication process. Thus, the observed calcium release corresponds to a release from the interior of the disks into the cytoplasm of the rod outer segment. The partial-bleaching studies indicate a stoichiometric release and, although the absolute amount of calcium released varies with the precise experimental conditions, the maximal release is about one calcium for each rhodopsin bleached. A light-induced increase in the calcium permeability of rhodopsin containing phospholipid bilayers has been observed recently by Hong and Hubbell (personal communication); this finding supports the contention that the calcium release in our experiments is attributable directly to the presence of rhodopsin in the disk membrane.

Possible mechanisms for the release of calcium from the disks include a pore opening upon bleaching, a light-induced change in the affinity of a specific calcium-binding site on the external (cytoplasmic) side of the membrane, and a carrier ejecting calcium upon bleaching. If rhodopsin were a calcium pore that opened upon light exposure, then under our experimental conditions one would expect that the total amount of calcium released from the disk vesicles would not be directly proportional to the percent bleaching and that the kinetics would be faster for larger amounts of bleaching because more pores would be opened. The partial-bleaching experiments of Figure 2 show total calcium release that is proportional to the amount of rhodopsin bleached. This is confirmed by the calcium release seen upon bleaching the remaining rhodopsin in a partially bleached sample (Figure 3). The release is clearly stoichiometric. The observed kinetics are independent of the amount of rhodopsin bleached, but it is not clear whether our experiments distinguish the kinetics of the actual release process from the kinetics of washout from the filter holder. The amount of calcium released by a light-activated pore should depend upon the level of internal calcium—we see no such dependence. The second mechanism, that of a light-activated release of externally bound calcium, is eliminated by our experiments in which no release is seen from sonicated disks that have radioactive calcium only on the external surface and by the experiments with the calcium ionophores.

Our evidence most strongly suggests a "one-shot carrier" model in which, upon bleaching, each rhodopsin releases a single calcium from within the disk. Such a mechanism is consistent with all of our observations: release proportional to percent bleaching, release upon bleaching the remaining rhodopsin in a partially bleached sample, release independent of internal calcium level, and no release of externally-bound calcium. The recovery experiments based upon both the rhodopsin ΔA_{500} and protein analysis show that the release cannot be explained by a reduced binding of the disks to the glass particles upon bleaching and a subsequent loss of material from the filter. The fact that binding to the glass particles has little effect upon regenerability of the disks implies that the release of calcium is not due to a denaturation of rhodopsin upon bleaching.

We have been unable to obtain calcium release after sonication of bleached and regenerated disks. This remains an area for further study, but the implication is that simple regeneration of rhodopsin with 11-*cis*-retinal is insufficient to reload the release sites.

Further elucidation of the mechanism is obtained from the temperature dependence of the release, which is consistent with a process after the formation of metarhodopsin I being responsible for the release. This could be the metarhodopsin I to metarhodopsin II transition, which has been suggested as a key step in generation of the neural signal (Abrahamson and Ostroy, 1967) and has also been linked in a preliminary report to calcium release (Nöll, 1974). The temperature effect could also involve other factors, such as the fluidity of the membrane. The pH profile of the release suggests tht a group with a pK between pH 7.0 and 8.0 is involved. Such groups include the sulfhydryl groups of rhodopsin and, interestingly, the Schiff base linkage as measured in squid metarhodopsin (Hara and Hara, 1972; Hubbard and St. George, 1958).

It has been estimated that at least 100 transmitter molecules need to be released for each rhodopsin bleached in order to block sufficient sodium channels to give the observed hyperpolarization of the plasma membrane (Cone, 1973; Yoshikami and Hagins, 1973). If this is so, our results would suggest either that there is another intermediate step after calcium release, in which amplification is attained, or that under physiological conditions a combination of circumstances exists that permits a larger release than is seen in our experiments. Our experiments do not resolve the kinetics of release on the millisecond time scale of the physiological process.

These findings suggest that rhodopsin can be conveniently classed neither as a pore nor as a carrier. Our interpretation is that the photoisomerization of the retinal chromophore induces a conformational change in rhodopsin that results in the translocation of calcium from an internal binding site to the space outside of the disk. It is not known whether this site is loaded before or after bleaching. As in the usual picture of a membrane pore or channel, the main structure of the protein does not change its orientation in the membrane; however, unlike a pore or channel, only one ion is translocated for each activation. Rhodopsin thus falls between the usual views of pores and carriers and, although we have chosen to call it a "one-shot carrier", a "one-shot pore" would be equally accurate.

Acknowledgments

The authors acknowledge the generosity of the following persons: Gene W. Stubbs, who constructed the transparent filter holder that made the bleaching experiments possible; Dr. Howard Kutachi, who allowed us to monopolize his liquid

scintillation counter; W. E. Scott of Hoffman-La Roche, Inc., who provided the 11-*cis*-retinal and the ionophore X-537A; and Dr. R. J. Hosley of Eli Lilly and Co., who provided the ionophore A23187. We also thank Dr. Wolfgang Haller of the National Bureau of Standards and Phillip Sliney of Corning Glass Co. for valuable suggestions on the use of glass beads.

References

Abrahamson, E. W., and Ostroy, S. E. (1967), *Prog. Biophys. Mol. Biol. 17,* 179.

Bownds, D., and Brodie, A. E. (1975), *J. Gen. Physiol. 66,* 407.

Bownds, D., Gordon-Walker, A., Gaide-Huguenin, A. C., and Robinson, W. (1971), *J. Gen. Physiol. 58,* 225.

Cohen, A. I. (1972), *Handb. Sens. Physiol., Part 2 7,* 63.

Cone, R. A. (1973), in Biochemistry and Physiology of Visual Pigments, Langer, H., Ed., Berlin-Heidelberg-New York, Springer-Verlag, pp 275ff.

Hagins, W. A. (1972) *Annu. Rev. Biophys. Bioeng. 1,* 131.

Hagins, W. A., and Yoshikami, S. (1974), *Exp. Eye Res. 18,* 299.

Hara, T., and Hara, R. (1972), *Handb. Sens. Physiol. Part 1 7,* 720.

Hemminki, K. (1975a), *Vision Res. 15,* 69.

Hemminki, K. (1975b), *Acta Physiol. Scand. 95,* 117.

Hendricks, Th., Daeman, F. J. M., and Bonting, S. L. (1974), *Biochim. Biophys. Acta 345,* 468.

Hubbard, R., and Kropf, A. (1958), *Proc. Natl. Acad. Sci. U.S.A. 44,* 130.

Hubbard, R., and St. George, R. C. C. (1958), *J. Gen. Physiol. 41,* 501.

Korenbrot, J. I., and Cone, R. A. (1972), *J. Gen. Physiol. 60,* 20.

Liebman, P. A. (1974), *Invest. Opthalmol. 13,* 700.

Litman, B. J. (1974), *Biochemistry 13,* 2844.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Mason, W. T., Fager, R. S., Abrahamson, E. W. (1974), *Nature (London) 247,* 562.

Mason, W. T., and Lee, Y. F. (1973), *Nature (London), New Biol. 244,* 143.

Neufeld, A. H., Miller, W. H., and Bitensky, M. W. (1972), *Biochim. Biophys. Acta 266,* 67.

Nöll, G. (1974), Ph.D. Thesis, Aachen, as referenced by H. Stieve (1974) in Biochemistry of Sensory Functions, Jaenicke, L., Ed., New York-Heidelberg-Berlin, Springer-Verlag, p 79.

Poo, M. M., and Cone, R. A. (1973), *Exp. Eye Res. 17,* 503.

Sillman, A. J., Ito, H., and Tomita, T. (1969), *Vision. Res. 9,* 1443.

Smith, H. G., Stubbs, G. W., and Litman, B. J. (1975), *Exp. Eye Res. 20,* 211.

Sorbi, R. T., and Cavaggioni, A. (1975), *Biochim. Biophys. Acta 394,* 577.

Stubbs, G. W., Smith, H. G., and Litman, B. J. (1976), *Biochim. Biophys. Acta 426,* 46.

Szuts, E. Z., and Cone, R. A. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1471.

Tomita, T. (1972), *Handb. Sens. Physiol. Part 2 7,* 483.

Wald, G. (1968), *Nature (London) 219,* 800.

Weller, M., Virmaux, N., and Mandel, P. (1975), *Nature (London) 256,* 68.

Wormington, C. M., and Cone, R. A. (1975), *Biophys. J. 15,* 171a.

Yoshikami, S., and Hagins, W. A. (1973), in Biochemistry and Physiology of Visual Pigments, Langer, H., Ed., New York-Heidelberg-Berlin, Springer-Verlag, p 245.