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Photoinduced Calcium Release from Rhodopsin–Phospholipid Membrane Vesicles†

Patricia N. Tyminski, Richard T. Klingbiel, Rebecca A. Ott, and David F. O'Brien*

ABSTRACT: Brief blue-green light exposure of rhodopsin–phospholipid membrane vesicles that contained divalent cations released the cations from the vesicles. The photoinduced release is due to an increase in permeability of the membrane. The quantity of ions released depends on the initial ionic concentration inside the vesicles. At 37 °C and an internal concentration of 30 mM Ca^{2+}, the initial flux for rhodopsin–egg phosphatidylcholine membrane vesicles was 0.25 ± 0.11 Ca^{2+} per bleached rhodopsin per s. Similar fluxes were observed for the release of Co^{2+}, Mn^{2+}, Ni^{2+}, and Mg^{2+}. The addition of proton uncouplers and lipophilic anions accelerated the rate to ~1 Ca^{2+} per bleached rhodopsin per s. The flux was independent of the concentration of rhodopsin in the membranes and sensitive to the head-group composition of the rhodopsin–phospholipid vesicles. Analysis of the fraction of Ca^{2+} released and the fraction of bleached rhodopsin per vesicle showed that a single bleached rhodopsin per vesicle is necessary and sufficient for Ca^{2+} release. Ca^{2+} release was not observed from thermally bleached rhodopsin. These results are discussed with regard to the possible role of Ca^{2+} as an excitatory transmitter in vision.

Light absorption by the 11-*cis*-retinal chromophore of rhodopsin isomerizes the chromophore and initiates visual excitation (Wald, 1968). Excitation of the rod cell causes a hyperpolarization of its potential (Tomita, 1965; Bortoff & Norton, 1965), because of a reduction of the rod outer segment (ROS)[1] plasma membrane permeability (Hagins, 1972; Korenbrot & Cone, 1972). It is generally accepted that a transmitter is necessary to mediate between the light-activated rhodopsin in the disk membranes of the ROS and the site of the sodium permeability in the plasma membrane (Baylor & Fuortes, 1970).

Hagins (1972) proposed that the transmitter is calcium ion that is released from the disks into the ROS cytoplasm on light exposure of rhodopsin. In electrophysiological experiments, the addition of calcium to a retina mimicked the effect of light (Yoshikami & Hagins, 1973; Hagins & Yoshikami, 1974; Brown et al., 1977; Lipton et al., 1977), and the addition of EGTA to a retina attenuated the effect of light (Hagins & Yoshikami, 1977; Brown et al., 1977). Osmotic measurements on isolated ROS's demonstrated that the addition of calcium to the buffer reduces the sodium permeability of the plasma membrane (Bownds & Brodie, 1975; Wormington & Cone, 1978) and depresses the cyclic GMP levels in the ROS (Woodruff & Bownds, 1979). It has been demonstrated that calcium is present in sufficient quantities inside the disk membranes to serve as the transmitter (Liebman, 1974; Szuts & Cone, 1977). Although these data support the hypothesis that calcium is the transmitter in ROS membranes, clear evidence for the photorelease of calcium from disk membranes in sufficient quantities and with the proper time course has yet to be achieved. It has been estimated that $10–10^3$ transmitter molecules per bleached rhodopsin need to be released in $\sim 10^{-1}$ s (Cone, 1973; Yoshikami & Hagins, 1973).

Several attempts to measure the photoinduced release of calcium from various ROS disk preparations have been reported. Many were summarized by Smith et al. (1977), who reported that the photorelease of $^{45}Ca^{2+}$ from sonicated ROS disks occurred with a yield of 0.75 Ca^{2+} per bleached rhodopsin at high levels of bleaching (24–100%). Smith & Bauer (1979) found somewhat larger releases at lower bleach levels (~2 Ca^{2+} per bleached rhodopsin at 5% bleaching), but the release required $\sim 10^2$ s. Szuts & Cone (1977) evaluated the photorelease of endogenous calcium from freshly isolated dark-adapted frog ROS disks by atomic absorption analysis and did not observe a significant release within a few seconds at low bleach levels (0.01% bleaching).

Photoinduced permeability change of rhodopsin–phospholipid membrane vesicles has also been reported (O'Brien et al., 1977b; Hubbell et al., 1977; Darszon et al., 1977; O'Brien, 1979; Gold & Korenbrot, 1979). Light exposure of rhodopsin–phospholipid membrane vesicles, which are closed unilamellar bilayers, increases the permeability of the vesicles to divalent cations without destroying the bilayer (O'Brien, 1979).

† From the Research Laboratories, Eastman Kodak Company, Rochester, New York 14650. *Received May 12, 1981; revised manuscript received October 6, 1981.*

[1] Abbreviations: ROS, rod outer segment; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; cyclic GMP, guanosine cyclic 3′,5′-phosphate; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; Pipes, piperazine-*N,N'*-bis(2-ethanesulfonic acid); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; DOPC, dioleoylphosphatidylcholine; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; meta I, metarhodopsin I; meta II, metarhodopsin II; Rh, rhodopsin; BRh, bleached rhodopsin; ND, neutral density; GTPase, guanosinetriphosphatase.

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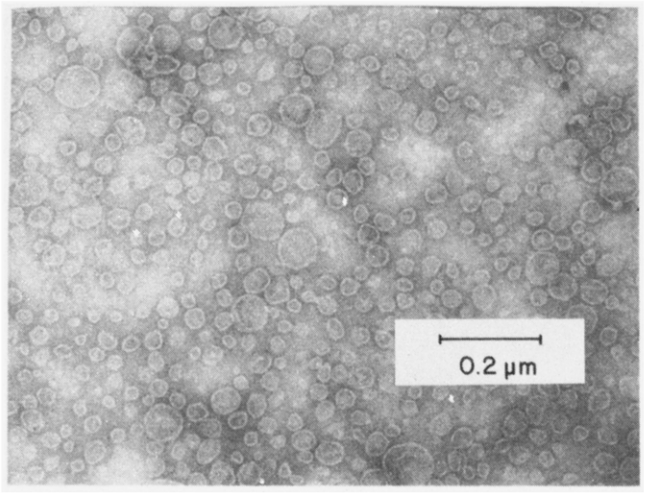

FIGURE 1: Electron micrograph of sonicated rhodopsin–egg PC membrane vesicles after osmium tetroxide fixation and ammonium molybdate negative stain.

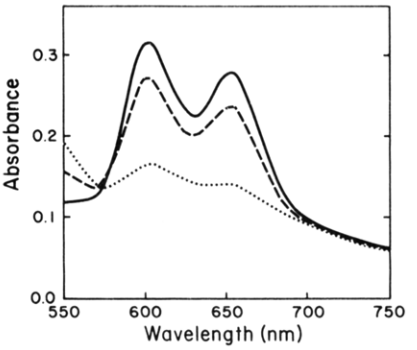

FIGURE 2: Absorption spectra of arsenazo III dye with an aqueous suspension of rhodopsin–egg PC (1:500) membrane vesicles which initially contained 30 mM $CaCl_2$, 100 mM NaCl, and 40 mM Hepes vs. a sample of the dye alone. The sample had 4.0 μM rhodopsin in 1.0 mL of 150 nM NaCl and 40 mM Hepes, pH 6.8, buffer at 37 °C. The spectra show the sample before flash exposure (···), after an initial flash bleach, which increases the concentration of the Ca^{2+}–arsenzo III complex (flash-released Ca^{2+}) (- - -), and after total bleach (total light-released Ca^{2+}) (—).

The quantity of ions released is proportional to the initial concentration of entrapped ions in the vesicle. At low levels of rhodopsin bleaching (1–2 rhodopsins per vesicle), high yields (40–160) of photoreleased Ca^{2+} per bleached rhodopsin were observed. These yields are significantly greater than those reported for sonicated ROS disks and are similar in quantity to that needed for the Ca^{2+} transmitter hypothesis of visual excitation, provided the rate of release is fast enough. The present report describes our findings on the rate of photorelease of Ca^{2+} from rhodopsin–phospholipid membrane vesicles.

Materials and Methods

General Procedures. Frozen, dark-adapted bovine retinae were obtained from George A. Hormel Co. or American Stores Packing. Egg PC, egg PE, and DOPC were obtained from Sigma Chemicals, and bovine PS was obtained from Supelco Inc. The lipid purity was evaluated by thin-layer chromatography, and where necessary, the lipids were purified by silicic acid chromatography. Xylenol orange dye was obtained from Kodak Laboratory Chemicals. Arsenazo III dye was purchased from Aldrich Chemical Co. and purified by the method of Kendrick (1976).

All rhodopsin experiments were performed under dim red light (Kodak safelight filter no. 1) or in complete darkness. The procedures for isolation of bovine rod outer segments, purification of rhodopsin in detergent, and preparation of rhodopsin–phospholipid membrane vesicles were described by Hong & Hubbell (1973) and O'Brien et al. (1977a).

Photoinduced Ion Release. Rhodopsin–phospholipid membrane vesicles were concentrated to 10–60 mM lipid by centrifugation or ultrafiltration. The vesicles in 10 mM buffer and 1 mM EDTA were mixed with an aliquot of salts to yield the desired ionic concentration. In a typical experiment, the vesicle suspension in 100 mM NaCl, 30 mM $CaCl_2$, and 40 mM Hepes, pH 7.0, was loaded by sonication under argon in an ice bath with a sonic probe. The resultant preparation consisted of unilamellar vesicles, as shown by negative-stain (ammonium molybdate) electron microscopy. The electron micrographs (Figure 1) show >90% of the images with diameters between 250 and 600 Å with an average size of the dried vesicle images of 440 Å. The free Ca^{2+} was removed by passing the vesicles through a Bio-Gel P-6 column at 4 °C in the presence of isoosmolar sodium and/or potassium salts in the same buffer. The procedures of preparation, concentration, sonication, and gel permeation chromatography were accomplished with high-recovery yields of rhodopsin for each step (O'Brien, 1979). Appropriate amounts of rhodopsin–lipid membranes were combined with isoosmolar buffer and metallochromic indicator dye for the ion-release experiment. Arsenazo III (35–75 μM) was used for calcium analysis, and xylenol orange (75 μM) was used for cobalt, manganese, and nickel analyses. The absorption spectrum of a 1.0-mL sample of the sonicated, loaded, and desalted rhodopsin–lipid membrane vesicles was obtained vs. a solution of the dye alone in a thermostated Cary 118 spectrophotometer (Figure 2). After a dark spectrum was obtained, the sample was flashed with a Strobonar photoflash (1 ms) through a Corning CS 5-57 filter (340–540 nm). The flash was attenuated by selected neutral density filters to control the extent of rhodopsin bleaching. Immediately after the flash, the absorbance increase at 650 nm (arsenazo III) or 585 nm (xylenol orange) was recorded continuously (Figures 3–5). In a normal experiment, the release was followed for ~1000 s until the absorbance had reached a steady value. A new absorbance spectrum was obtained to determine the increase in M^{2+}–dye concentration, and therefore the flash-released Ca^{2+} (Figure 2). The sample was then completely bleached with room lights, and a spectrum was obtained for total light-induced release of the ion. In some cases, the Ca^{2+} was released with the ionophore A23187. Finally, the 1.0 mL of membrane suspension was dissolved with 0.1 mL of 5% Triton X-100 surfactant to determine the total concentration of divalent cations in the preparation. Triton X-100 did not contribute to the measured M^{2+}–dye concentration. The change in concentration of the M^{2+}–dye complex was calibrated by addition of standard amounts of M^{2+}.

The ratio of the flash-released Ca^{2+} to the total light-released Ca^{2+} is the fraction of flash-released Ca^{2+}, *F*. The total light-released Ca^{2+} plus any Ca^{2+}–dye complex (dark Ca^{2+}) present before the sample was exposed to the Strobonar flash usually amounted to >0.95 of the total Ca^{2+} in the sample, which was determined by the concentration of Ca^{2+}–dye complex after Triton X-100 lysis of the membranes or by treatment with the ionophore A23187 (O'Brien, 1979). Therefore, only minor amounts of Ca^{2+} were bound to the rhodopsin–egg PC membrane vesicles.

The degree of rhodopsin bleaching was determined directly from the absorption spectrum in the xylenol orange experiments, since the isosbestic point of the free dye and dye–cation complex is near 490 nm (O'Brien, 1979). Rhodopsin bleaching in the arsenazo III experiments was determined by exposing

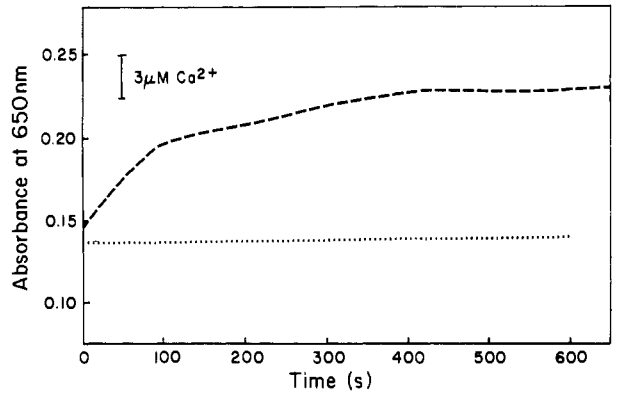

FIGURE 3: Flash-induced Ca^{2+} release determined by the absorbance increase for the Ca^{2+}–arsenazo III complex at 650 nm vs. time. The sample is the same as in Figure 2. The lower trace is the absorbance for the sample before flash exposure. The calibration bar shows the increase in Ca^{2+} for a given absorbance increase of the Ca^{2+}–arsenazo complex at 650 nm.

identical membrane samples with and without Ca^{2+} to the same bleaching flashes. The decrease in absorbance at 500 nm in the absence of Ca^{2+} was due to the rhodopsin bleaching.

Results and Discussion

Membrane vesicle preparations used in these studies consisted of purified delipidated dark-adapted bovine rhodopsin recombined by detergent dialysis with purified phospholipids. The most commonly used lipid was egg phosphatidylcholine in a molar ratio of 1 rhodopsin to 500 lipids, which will be represented as rhodopsin–egg PC (1:500). An electron micrograph of the sonicated vesicles is shown in Figure 1. The average size of the dried vesicle images is 440 Å and must be corrected to give the average size of the vesicle in aqueous suspension. A correction factor of 0.75 (Olson et al., 1979) yields an average outside diameter of the rhodopsin–egg PC (1:500) membrane vesicles of 330 Å. The entrapped volume of the vesicles was determined with [^{14}C]inulin (O'Brien, 1979). These data yielded an inside diameter of 250–290 Å. In addition, the ratio of external to internal lipid head groups was measured by NMR spectroscopy with the aid of shift and relaxation reagents (O'Brien et al., 1977b). The lipid fraction on the exterior surface of the membrane vesicles was 0.66 ± 0.02. These three independent estimates yielded an average external diameter of 340 ± 20 Å for rhodopsin–egg PC (1:500) membrane vesicles. The number of rhodopsin molecules per vesicle of this diameter is 15 ± 2.[2]

We have previously reported that the quantity of ions photoreleased is dependent on the concentration of the ion of interest (O'Brien, 1979). Most of the present experiments used

Table I: Light-Induced Ca^{2+} Flux for Rhodopsin-Phospholipid (1:500) Membranes in 150 mM NaCl–40 mM Hepes Buffer at 37 °C

membrane	pH	bleached rhodopsin (μM)	flux [Ca^{2+}/(BRh·s)]
Rh-egg PC	6.81	0.50	0.40
	7.00	0.21	0.26
	6.95	0.11	0.10
	6.92	0.24	0.33
	6.91	0.29	0.20
	6.96	0.22	0.21
			av 0.25 ± 0.11
Rh-DOPC	6.78	0.27	0.30
	6.95	0.39	0.17

Table II: Light-Induced Co^{2+} Flux for Rhodopsin-Phospholipid (1:500) Membranes in 150 mM NaCl–40 mM Hepes Buffer, pH 7

membrane	temp (°C)	Co^{2+} (mM)	flux [Co^{2+}/(BRh·s)]
Rh-egg PC	37	30	0.37
Rh-DOPC	37	30	0.42
Rh-egg PC	25	30	0.12
	25	30	0.17
	25	30	0.11
	25	100	0.17
Rh-DOPC	25	30	0.08

30 mM Ca^{2+} or Co^{2+}. The ratio of calcium to rhodopsin in ROS disk membranes was found to be 0.2 (Liebman, 1974) and 0.1–0.2 (Szuts & Cone, 1977). At these ratios, if the calcium were free within the disk, the intradiskal calcium concentration would be 5–10 mM. In our experiments, the higher than native calcium concentration was used in order to load each small vesicle with a substantial number of ions (130–150) for the ion-release experiment.

An example of light-induced release of calcium from rhodopsin–egg PC (1:500) membrane vesicles initiated by a brief flash exposure (1 ms) is shown in Figure 3. Immediately after the flash, the 650-nm recording trace was started, which created a 3–5-s delay from flash to the first record. Interaction between Ca^{2+} and arsenazo III occurred during the delay and increased in magnitude over the time of the recording. No detectable latency was observed on this time scale. Also note that there was minimal interaction between the Ca^{2+} in the unexposed vesicles and the exterior arsenazo III at 37 °C for more than 10 min. The initial light-induced flux of Ca^{2+} from the vesicles varied from about 0.1 to 1.0 ion per bleached rhodopsin per second (BRh·s). Various factors affect the flux and are described below. Values for the light-induced flux were estimated from the initial rate of cation release, determined by the extent of the cation–dye reaction and the degree of flash-induced rhodopsin bleaching.

The calculated initial light-induced flux of calcium is given in Table I for six separate preparations of rhodopsin–egg PC (1:500) membrane vesicles. The average flux was 0.25 ± 0.11 Ca^{2+}/(BRh·s). In each of these experiments, the external cation was sodium, and the anion was chloride. The pH was near 7.0, although the measured pHs varied somewhat, as shown. The pH before and after each experiment was the same within ±0.03 pH unit with either 40 or 80 mM buffer. The source of the variation in the flux data is not well understood, but it is likely due to a variation in the rhodopsin preparations. Repeated ion-release experiments with aliquots of the same membrane preparation varied by ±10%. For evaluation of the effect of different factors on the light-induced release of cations, comparisons were made between membrane vesicles formulated from the same rhodopsin preparation.

[2] Knowledge of the surface area occupied by rhodopsin and phosphatidylcholine molecules allows an estimate of the number of rhodopsins for 340-Å vesicles. The average area per phospholipid is 70 Å^2 (Small, 1967). Recent X-ray scattering data suggest that rhodopsin is a cylinder 31 Å in diameter and 97 Å long (Sardet et al., 1976). Dratz et al. (1979) estimate that rhodopsin is a cylinder in the hydrocarbon region of the bilayer with a radius of 16.7 ± 2.5 Å. For our calculations, we assume rhodopsin to be a transmembrane cylinder (Fung & Hubbell, 1978; O'Brien et al., 1977b; Sardet et al., 1976) and that it occupies equal circular areas on each side of the membrane with a radius of 16 Å or an area of 800 Å^2. Thus, the area occupied by 1 unit of rhodopsin and 500 PC molecules is 36 600 Å^2. A vesicle with an exterior diameter of 340 Å will have an interior diameter of 250 Å with a bilayer thickness of 45 Å (Huynh, 1973); therefore, the total surface area is 5.6×10^5 Å^2. A 340-Å vesicle will contain 15.3 rhodopsin molecules in random orientation and 7600 phospholipid molecules. Rhodopsin molecules occupy 3.4% of the exterior surface area.

Table II gives data for the release of Co^{2+} from rhodopsin–egg PC vesicles. At 37 °C, the flux is similar to that observed for Ca^{2+}. The first entries in Tables I and II can be directly compared, since the same rhodopsin–egg PC (1:500) preparation was used, with Ca^{2+} in one case and Co^{2+} in the other. Within the error of the experiment, the release rates of Ca^{2+} and Co^{2+} were the same. Similar release rates were observed in single experiments for Mg^{2+}, Ni^{2+}, and Mn^{2+}. In comparison to these data for the light-induced flux of divalent cations, we observed previously (O'Brien et al., 1977b) that the Eu^{3+} permeability is about an order of magnitude less [$\sim$0.01 Eu^{3+}/(BRh·s)].

The observed flux is significantly smaller than expected if the vesicle is a sealed sphere in which a hole or channel is formed by the action of light. The calculated flux of released ions in this case can be obtained by assuming a model wherein ions are released through rigid cylindrical channels against an electrochemical potential. The flux is measured in terms of the number of moles of ions released per second per square centimeter channel cross-sectional area. The calculated flux for 30 mM Ca^{2+} in a vesicle of 300-Å interior diameter, with 100 mM NaCl on the inside and 150 mM NaCl on the outside, with one channel (pore) 40 Å long and 10 Å in diameter was 1.2×10^6 Ca^{2+}/(pore·s) at 35 °C. This value is only modestly affected by electrodiffusion corrections and reduced by a factor of $\sim$5 by adsorption of ions and surface potential. Ion–channel interactions, the Renkin effect, reduce the estimated flux to 1.3×10^5 Ca^{2+}/(pore·s).

If the ion must overcome an activation barrier to transit the pore, the expected flux is reduced in an Arrhenian fashion. If the permeating ion introduces a net charge into the channel interior surrounded by a low dielectric medium, the activation barrier is due to dielectric exclusion. Parsegian (1969) treated the case for a channel whose length is far greater than its radius. Levitt (1978) has modified the calculation for cases where the channel length and diameter are comparable to our case. Adjustment of our flux estimate for Ca^{2+} by the Boltzmann factor yields 41 Ca^{2+}/(pore·s) for the largest barrier of 10.3 kT. Therefore, even for the maximum reasonable dielectric exclusion, the observed fluxes given in Tables I and II are $\sim$0.01 that predicted for a channel in a vesicle.

At nearly equal concentrations of Ca^{2+} carrier and bleached rhodopsin, the ionophore is more effective at Ca^{2+} release. The addition of 1 μL of 0.4 mM A23187 calcium ionophore in ethanol to 1.0 mL of a suspension of rhodopsin–egg PC (1:500) membranes produced a rapid release of Ca^{2+} from the interior of the vesicles. The release was complete in 50 s at 37 °C. For comparison, an identical membrane preparation (4.5 μM rhodopsin) was optically bleached with a 1-ms flash to yield 0.6 μM bleached rhodopsin. The initial rate of Ca^{2+} release by 0.6 μM bleached rhodopsin was 0.1 that attained in the presence of 0.4 μM Ca^{2+} ionophore. The permeability pathway formed by light exposure of rhodopsin–egg PC membrane vesicles permits a Ca^{2+} flux 10^{-1} that of the ionophore A23187 and 10^{-2}–10^{-5} that estimated for a channel. The difference between the observed and the predicted flux prompted an examination of the experiment for factors which might produce an artifactually slow Ca^{2+} release.

Effect of Lipid Composition. Rhodopsin–phospholipid membranes were prepared from egg PC, dioleoyl-PC, egg PC/cholesterol (90/10), egg PC/egg PE (50/50), and egg PC/egg PE/bovine PS (45/45/10) to evaluate the effect of lipid composition on the light-induced Ca^{2+} release. Variation in the fatty acid composition of phosphatidylcholine from egg PC to DOPC does not significantly change the observed

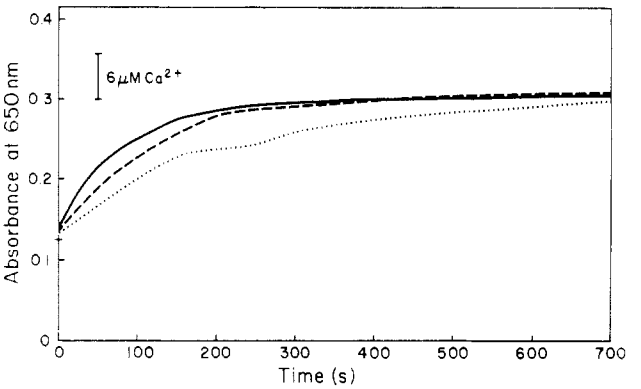

FIGURE 4: Effect of uncoupler CCCP on the light-induced Ca^{2+} release determined by the absorbance increase at 650 nm vs. time for an aqueous suspension of rhodopsin–egg PC (1:500) membrane vesicles with arsenazo III dye. The sample contained 4.6 μM rhodopsin in 1.0 mL of 150 mM NaCl and 40 mM Hepes, pH 7.0, buffer at 37 °C. The flash exposure bleached 0.24–0.29 μM rhodopsin in the different experiments. The traces are shown for different concentrations of the uncoupler CCCP: none (···); 1 μM (- - -); 10 μM (—).

light-induced flux of Ca^{2+} or Co^{2+} from the vesicles at 25 and 37 °C (Tables I and II). The rate of ion release is insensitive to the presence of 10 mol % cholesterol in the membrane but is very sensitive to the head-group composition. The measured flux for a pair of membranes prepared at pH 7.4 in 80 mM Hepes buffer was 0.15 Ca^{2+}/(BRh·s) for rhodopsin–egg PC (1:500) and 0.02 Ca^{2+}/(BRh·s) for rhodopsin–egg PC/egg PE (1:250/250). The Ca^{2+} flux was independent of whether Na^+ or K^+ was the exterior cation. The Ca^{2+} release from a rhodopsin–PC/PE/PS (225/225/50) membrane [head-group composition similar to that of ROS disk membranes (Crain et al., 1978)] was the same as the release from rhodopsin–egg PC/egg PE.

Effect of Ionophores. The addition of lipophilic anions or proton uncouplers accelerates the release of photoinduced Ca^{2+}. In experiments with thiocyanate rather than chloride and with 1–10 μM carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), the Ca^{2+} flux increased by factors of two to five. A rhodopsin–egg PC (1:500) membrane (4.6 μM rhodopsin) was presented with a light flash which bleached 6% of the rhodopsin, and the initial Ca^{2+} flux was 0.33 Ca^{2+}/(BRh·s). The addition of 1 and 10 μM CCCP increased the flux for the same membrane and the same exposure conditions to 0.57 and 1.2 Ca^{2+}/(BRh·s), respectively (Figure 4). In the presence of CCCP, the rate of light-induced Ca^{2+} release from rhodopsin–egg PC (1:500) membrane vesicles approaches the rate of Ca^{2+} ionophore induced release mediated by A23187. When a rhodopsin–egg PC (1:500) membrane was prepared in 100 mM NaSCN rather than sodium chloride, the observed Ca^{2+} released from the vesicles was increased by at least a factor of two. The effects of both SCN^- and CCCP are probably due to facilitation of proton movement across the rhodopsin–egg PC membrane to maintain electroneutrality during Ca^{2+} exodus from the vesicles.

Replacement of the exterior sodium ions with less permeable ions did not greatly alter the light-induced flux. The substitution of choline for sodium resulted in a Ca^{2+} flux of 0.19 Ca^{2+}/(BRh·s) compared to 0.28 Ca^{2+}/(BRh·s).

The replacement of chloride by sulfate in experiments with rhodopsin–egg PC (1:500) membranes and cobalt ion reduced the Co^{2+} flux to 0.3 that normally observed in the presence of chloride. This effect is similar to the difference in permeability of chloride and sulfate in ROS disk membranes (Amis et al., 1981) and suggests the anion movement may be important in these experiments.

Table III: Effect of Rhodopsin-Egg PC Ratio on Light-Induced Ca^{2+} Flux

Rh–egg PC mol ratio	fraction of Rh bleached	bleached Rh per vesicle	flux [Ca^{2+}/(BRh·s)]
1:500	0.050	0.77[a]	0.21
	0.081	1.24[a]	0.21
1:2500	0.32	1.12[b]	0.20
	0.38	1.33[b]	0.20

[a] Based on 15.3 rhodopsins per vesicle. [b] Based on 3.5 rhodopsins per vesicle.

Effect of Rhodopsin Concentration. A major difference between the rhodopsin–lipid membrane vesicles used in this study and ROS membranes is the concentration and orientation of the rhodopsin in the membrane. In ROS membranes, the rhodopsin:lipid ratio is 1:65 (Dratz et al., 1979), and the rhodopsins are all oriented with the amino terminus in the interior of the disk membrane (Hargrave, 1977; Adams et al., 1978), whereas in the reconstituted membranes used in these studies the rhodopsin orientation is randomized because of the nature of the self-assembly of the protein and lipid during detergent dialysis (Chen & Hubbell, 1974; Fung & Hubbell, 1978). In addition, we generally have used 1:500 or 1:1000 rhodopsin:lipid membranes. Therefore, the frequency of rhodopsin–rhodopsin interactions is less in the reconstituted preparations than in the ROS membranes. The presently available evidence suggests that rhodopsin is monomeric in the dark in ROS membranes (Chabre, 1975; Blaurock, 1977). Whether it remains so in the light is unclear. Montal et al. (1977) have suggested that a light-activated rhodopsin interacts with nonexposed rhodopsin molecules to form a channel for Ca^{2+} efflux from ROS disks. If such an interaction is required for the release of Ca^{2+} from membrane vesicles, the observed rate would be slower from rhodopsin–lipid (1:500) membranes than from the natural system. The lipid surface area per rhodopsin molecule will be 7 times as great in a 1:500 membrane as in the ROS membranes. The frequency of rhodopsin–rhodopsin interactions in 1:500 membranes will be 0.14 that found in ROS membranes, assuming the rate of lateral diffusion is the same in both cases. Since approximately one-third of the rhodopsins in the 1:500 membrane vesicles are in the retrograde orientation [determined by the proteolysis procedure of Fung & Hubbell (1978)], the frequency of interaction of rhodopsin molecules in the same orientation in 1:500 membranes will be ≤ 0.10 that found in ROS membranes.

One method of testing whether the frequency of rhodopsin–rhodopsin interactions is important for Ca^{2+} release is to prepare membranes with different rhodopsin concentrations and measure the Ca^{2+} flux under experimental conditions that bleach the same number of rhodopsins in each membrane preparation. A pair of membranes were prepared from the same rhodopsin under identical conditions, rhodopsin–egg PC (1:500) and rhodopsin–egg PC (1:2500). In the 1:500 and 1:2500 membranes, the frequency of rhodopsin–rhodopsin interactions should vary by a factor of five. The data for experiments with the 1:500 membrane and with the 1:2500 membrane are shown in Table III. Two bleaching levels were used in each experiment to give a range of bleaching of 0.7–1.4 bleached rhodopsins per vesicle. These numbers are based on the calculated number of 15.3 rhodopsins per vesicle for a rhodopsin–egg PC (1:500) membrane and 3.5 rhodopsins per vesicle for the 1:2500 preparation. Note that the observed Ca^{2+} flux is the same for each bleach level used with a given membrane, and the Ca^{2+}/(BRh·s) is very similar for both the 1:500 and the 1:2500 membranes. These data demonstrate

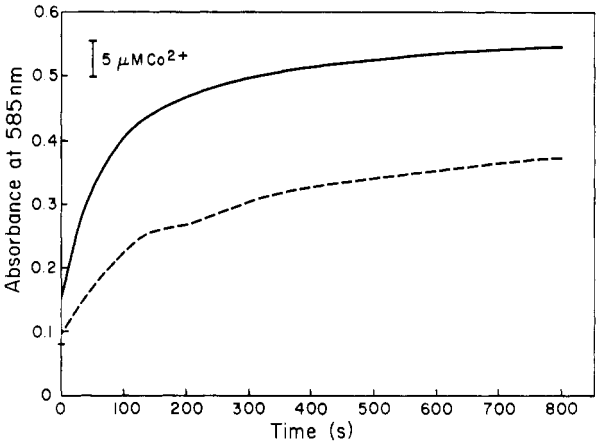

FIGURE 5: Light-induced Co^{2+} release determined by the absorbance increase at 585 nm vs. time of an aqueous suspension of rhodopsin–egg PC (1:500) membrane vesicles with xylenol orange dye. The sample contained 1.3 μM rhodopsin in 1.0 mL of 150 mM NaCl and 40 mM Hepes, pH 7.0, buffer. The Co^{2+} release is shown for two bleach levels: 10% (---); 45% (—).

that the Ca^{2+} flux is independent of the number of rhodopsins in the vicinity of the bleached rhodopsin or permeability pathway. Furthermore, if the rate of lateral diffusion of the rhodopsins in both membranes is similar, then the rhodopsin–rhodopsin interaction is not a controlling factor in the observed rate of Ca^{2+} efflux from the membrane vesicles.

The presence of rhodopsin interactions should also be reflected in the Ca^{2+} flux value at different levels of bleaching in the same membrane. As the number of bleached rhodopsins increases per vesicle, the initial rate of the release of Ca^{2+} will increase, but the Ca^{2+}/(BRh·s) will remain constant if the bleached rhodopsins do not need to interact with neighboring rhodopsins to form a permeability pathway. The flux is independent of the bleach level in Table III, and it is also in the data shown in Figure 5. The data are for Co^{2+} release from a rhodopsin–egg PC (1:500) membrane which was bleached through a 1.0 ND filter (10% bleach) in one run and was repeated with a flash without an ND filter (45% bleach) in the second experiment. The initial fluxes are very similar: 1.1 and 1.0 Co^{2+}/(BRh·s), respectively. These are the fastest release rates we have observed in the absence of a proton uncoupler. At the lower bleach level, there are approximately ten rhodopsins for every bleached rhodopsin per vesicle, whereas at the higher bleach level there are nearly equal numbers of bleached and unbleached rhodopsins per vesicle; therefore, initial Ca^{+} release depends primarily on the number of bleached rhodopsins and not on the number or concentration of neighboring unbleached rhodopsins.

Number of Bleached Rhodopsins per Vesicle To Initiate Ca^{2+} Release. The photoinduced release of ions reported here exhibits a finite retention of ions which can arise either from a fraction of vesicles having no bleached rhodopsin or as a result of a transient nature of the rhodopsin-mediated permeation upon bleaching. The statistical nature of both loading of ions and bleaching of rhodopsin allows an estimate of the fraction of vesicles which can be expected to have no bleached rhodopsin upon illumination of the sample and therefore constitute the fraction of unexposed vesicles which have effectively "trapped" their ions. This fraction can become appreciable only at a very low average number of bleached rhodopsins per vesicle. If there are on the average N rhodopsins per vesicle and the average fraction bleached is P, then Poisson statistics predict that the probability of there being exactly n rhodopsins bleached in a particular vesicle is $\Psi(n,PN)$, where

$$\Psi(n,PN) = \frac{(PN)^n e^{-PN}}{n!}$$

If we assume that only vesicles which have no bleached rhodopsin can keep their contents trapped, then the fraction trapped is simply $\Psi(n,NP)$ for $n = 0$, i.e., e^{-PN}, and the final fraction flash released is just $1 - e^{-PN}$.

Because arsenazo III dye absorbs strongly at the bleaching wavelengths, P is not constant throughout the sample, and the fractional flash release should be written as

$$F = \frac{1}{l}\int_0^l 1 - e^{-P(x)N}\,dx$$

where l is the sample path length and $P(x)$ the percent bleach at a distance x into the sample. For a sample obeying Lambert's law, $P(x) = \alpha e^{-\beta x}$, where β is the absorption coefficient and $\alpha = \beta l\bar{P}/(1 - e^{-\beta l})$ where $\bar{P}$ is the average percent bleach throughout the sample. Then the fractional release becomes

$$F = \frac{1}{l}\int_0^l 1 - e^{-\alpha N e^{-\beta x}}\,dx$$

which can be explicitly integrated to yield

$$F = -\sum_{n=1}^{\infty} \frac{(-1)^n}{nn!} \frac{(\beta l\bar{P}N)^n}{\beta l} \frac{1 - u^n}{(1 - u)^n}$$

where $u = e^{-\beta l}$. F was calculated from the above equation as a function of $\bar{P}N$ for experiments of measured $\bar{P}$ where the sample absorbance at 500 nm was $A = 1.48$. The value of N required for agreement between the computed and observed F values was then compared with the assayed estimate of N. The average value of flash-released Ca^{2+}, F, for nine experiments with Rh–egg PC (1:500) in 80 mM Hepes buffer, pH 7.35, at 37 °C was 0.71 ± 0.04, when the fraction of rhodopsin bleached ($\bar{P}$) was 0.14. From the above equation, these data give a value of N, the average number of rhodopsins per vesicle, of 16.6 ± 3.0. A second set of six experiments with a 0.08 fraction of rhodopsin bleached produced an average value of flash-released Ca^{2+} of 0.52 ± 0.06. This corresponds to 13.7 ± 3.8 rhodopsins per vesicle (N). These estimates agree within the experimental uncertainty of F with the average number of rhodopsins per vesicle (15.3 ± 2.1) deduced from the size of the vesicles and the rhodopsin to lipid content ratio. Agreement between these two independent estimates indicates that the observed retention of Ca^{2+} after the initial flash-induced release is due to a fraction of vesicles which do not have bleached rhodopsin. Thus, it is evident that a single bleached rhodopsin per vesicle can initiate the release. These results and the following observations indicate that the permeability pathway is the optically bleached rhodopsin.

Effect of Temperature. The effect of temperature on the light-induced release of Ca^{2+} was studied with a rhodopsin–DOPC (1:500) membrane at pH 7.6. The initial Ca^{2+} release/(BRh·s) was 0.35 at 37 °C, 0.10 at 25 °C, 0.024 at 9 °C, and only 0.002 at 4 °C. The variation in flux data with temperature is similar to the effect on the conversion rate of meta I to meta II for rhodopsin–DOPC (1:500) membranes (unpublished observations). The photorelease was essentially blocked at 4 °C. The predominant intermediate is metarhodopsin I at this temperature and pH (Matthews et al., 1963). A sample that was flashed at 4 °C and did not release Ca^{2+} was then warmed to 25 °C in the dark. It was replaced in the spectrophotometer compartment, and the absorbance was recorded at 4 °C. The absorbance at 650 nm increased after the sample was warmed in the dark, which shows that Ca^{2+} could be released by warming the sample after light exposure at 4 °C. Therefore, the Ca^{2+} release initiated by light absorption requires the activated rhodopsin to relax to at least the meta II intermediate.

Effect of Thermal Bleaching. Although light bleaching of rhodopsin–lipid membranes increases their permeability, we have found that thermal bleaching does not cause these membranes to lose entrapped divalent cations. In particular, a rhodopsin–DOPC membrane that was partially thermally bleached (~50%) was loaded with Ca^{2+} and prepared for ion-release studies in the manner described under Materials and Methods. The membrane vesicles successfully entrapped Ca^{2+} ions, and light exposure released the Ca^{2+}. The release was similar to those in membranes that contained dark rhodopsin without any thermally bleached rhodopsin. Membranes that have been partially light bleached will no longer entrap Ca^{2+}.

Time Variations in the Flux. The release rates shown in Figures 3–5 show small variations in the rate of release appearing as ripples on the release curves. These variations are seen on many of our release curves. They are often irreproducible with respect to amplitude among different preparations; however, they exhibit nearly the same periodicity and, in general, the amplitude of the variations decreases with increasing bleach level. It seems unlikely that the electrodiffusive release from individual vesicles would not have reached steady state in times comparable to the variations (hundreds of seconds), and, therefore, we interpret these changes as being indicative of a coupled transport of Ca^{2+} through the rhodopsin pathway. The theory of oscillatory transport across membranes from coupled transport is well understood in principle (Teorell, 1959; Katchalsky & Spangler, 1968; Glansdorff & Prigogine, 1971; Franck, 1978) if not mechanistically. It is important to realize that although the vesicles release their ions in a statistically independent fashion, after a simultaneous bleach, the collective time dependence of the release would still reflect the individual vesicle release. (The variations are not, for example, an artifact of the absorbance measurement due to mixing or thermal inhomogeneities.)

General Discussion

Brief blue-green light exposure of rhodopsin–phospholipid membrane vesicles that contain divalent cations releases the cations from the vesicles. Previous results (O'Brien, 1979) indicate that the photoinduced release of Ca^{2+} in these membrane vesicles is due to an increase in permeability of the membrane without disruption of the bilayer. The quantity of ions released is proportional to the initial ionic concentration in the vesicle, and high yields of photoreleased Ca^{2+} per bleached rhodopsin are observed when only a few rhodopsins per vesicle are bleached. The release can be inhibited by chemical modification of the rhodopsin prior to membrane formation (O'Brien et al., 1979). The present results provide valuable kinetic information about this light-induced release of Ca^{2+} and other ions from these membrane vesicles. At 37 °C, the observed average rate for Ca^{2+} release from rhodopsin–egg PC vesicles is 0.25 Ca^{2+}/(BRh·s). These flux data are similar to those reported by Gold & Korenbrot (1979) for rhodopsin–egg PC and sonicated ROS membranes. The flux is modestly increased by proton uncouplers and lipophilic anions; in some instances, the rate increases 5–fold. The initial Ca^{2+} flux is not strongly dependent on the concentration of rhodopsin molecules in the vesicles or on the number of bleached rhodopsins in the vesicle. A single bleached rhodopsin per vesicle is necessary and sufficient to initiate the Ca^{2+} release.

Consideration of mechanisms for the release of divalent cations from the membranes suggests three possibilities: a

light-induced change in cation binding to the rhodopsin, a light-induced opening of a channel or pore through the membrane, or light activation of a carrier to move ions across the vesicle wall. Our results are inconsistent with a change in the binding of Ca^{2+} to the vesicle exterior, since the addition of arsenazo III dye to the vesicles in the dark does not remove Ca^{2+} from the vesicles. Furthermore, experiments with the Ca^{2+} ionophore A23187 show that it depletes the supply of Ca^{2+} available for light release. These results are similar to those of Smith et al. (1977) for the light-activated release of Ca^{2+} from sonicated ROS disk membranes and agree with their conclusion that a change of Ca^{2+} binding is an unlikely mechanism for Ca^{2+} release. Classification of the release as a channel or carrier mechanism is more difficult. The Ca^{2+} release is dependent on the ionic concentration in the vesicle in a manner expected of channels. The lack of specificity in the rates of release of Ca^{2+}, Co^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} suggests a nonspecific channel rather than a carrier. However, the rate of release is much slower than expected for a channel and indeed approaches the rate of release for the Ca^{2+} ionophore A23187 only when the photorelease is done in the presence of an uncoupler. Smith et al. (1977) also had difficulty classifying the light-induced activity of rhodopsin as that of a channel or carrier and coined the term "one-shot" carrier to describe the behavior.

Whatever the mechanism of Ca^{2+} release in rhodopsin–phospholipid membrane vesicles, it is clearly not one shot. The number of ions photoreleased is large ($\sim$100) per bleached rhodopsin or permeability pathway. The observation of these large releases supports a role for Ca^{2+} as a transmitter in visual excitation. However, the rate of release reported here is too slow for Ca^{2+} from the disks to serve that function. The maximum observed initial flux of $\sim$1 Ca^{2+}/(BRh·s) is significantly less than the values calculated for the transmitter of 100–1000 Ca^{2+}/(BRh·s) (Cone, 1973; Yoshikami & Hagins, 1973). Photoinduced calcium release from the outer segments of photoreceptor cells in isolated retinae has been described (Gold & Korenbrot, 1980; Yoshikami et al., 1980). The observed release of at least 500 Ca^{2+} for each absorbed photon shows that light absorption by rhodopsin increases the calcium activity of the ROS cytoplasm. The source of the light-regulated calcium pool may be either the disk membranes as originally proposed by Hagins (1972) or the cytoplasmic binding sites (protein or membrane). The failure to observe high flux rates in rhodopsin–egg PC membranes could be due to the absence of some cofactor(s) necessary for fast Ca^{2+} release in ROS disks, or it could be representative of an inherent limitation of light-activated rhodopsin transmembrane release of calcium; in this case, the light-regulated calcium source of the ROS must be in the cytoplasm, as suggested by Szuts (1980).

In experiments with isolated sealed ROS's, Kaupp et al. (1979) observed the flash release in the presence of a Ca^{2+} ionophore of about 0.5 Ca^{2+} per bleached rhodopsin in 300 ms. Although the authors attribute the effect to a change in intradiskal Ca^{2+} binding rather than a permeability change of the disk, the magnitude of the transient flux is similar to those reported here. It may be that in the rhodopsin–egg PC membranes we observe an initial release of a few Ca^{2+} (1 or 2) per second as in the ROS, and then the Ca^{2+} release continues because the light-induced permeability change is more prolonged in the vesicles than in the ROS's. Smith & Bauer (1979) suggested that the permeability change in ROS disks is much shorter than in rhodopsin–egg PC vesicles. Our data suggest that the permeability increase is associated with the transition from meta I to meta II intermediates. A permeability increase associated with the meta II intermediate has also been suggested by Smith et al. (1977) from studies with ROS disks. The longer lifetime of the permeability pathway in rhodopsin–egg PC membranes may be associated with a longer lifetime of the meta II state in these membranes compared to ROS membranes. Unpublished observations from our laboratory show that rhodopsin–egg PC vesicles have a slower rate of meta II decay than rhodopsin in ROS membranes.

The measurement of physical phenomena with reconstituted membranes is always subject to difficulties associated with possible permanent perturbations of the protein. Because of the lack of general agreement on the nature of light-induced permeability changes in ROS disks, it is not possible to compare our results with an accepted standard of natural functional behavior. We have demonstrated that these membranes of rhodopsin and lipids behave photochemically and thermally very similarly to ROS membranes. In addition, they can be chemically regenerated (Hong & Hubbell, 1973) with 11-*cis*-retinal, and their circular dichroism spectra are similar to those of ROS membranes (Klingbiel et al., 1980). Recently we have successfully used these membranes with ROS GTPase and phosphodiesterase to reconstitute the light activation of phosphodiesterase activity at rates comparable to those of ROS membranes (Yee & Liebman, 1978). The rate of cyclic GMP hydrolysis is at least 10^3 times the Ca^{2+} flux measured for the same membrane. These data suggest that the slow rate of Ca^{2+} release in rhodopsin–phospholipid membrane vesicles is not likely due to a perturbation of the rhodopsin but either is a true representation of the natural system or is unnaturally slow because of the absence of some unrecognized ROS cofactor in the rhodopsin–phospholipid membrane vesicles.

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In Vitro Messenger Ribonucleic Acid Directed Synthesis and Processing of an Immunologically Identified Precursor to Tetradecapeptide Somatostatin from Bovine Hypothalamus†

Richard Ivell and Dietmar Richter*

ABSTRACT: mRNA isolated from bovine hypothalami was used to direct the in vitro synthesis of a precursor to the tetradecapeptide somatostatin. When a rabbit reticulocyte lysate translation system supplemented with [^{35}S]cysteine was used, a protein of apparent molecular weight 15 500 was identified as preprosomatostatin by reaction with specific rabbit antibodies. Cotranslational addition of dog pancreas microsomal membranes yielded an unglycosylated pro form of 14 500 daltons, implying the removal of a short signal sequence.

The tetradecapeptide somatostatin (SRIF)[1] has been suggested to function not only as a hormone but also as a neurotransmitter and as a local regulatory agent (Schally, 1978; Guillemin, 1978; Cohn & Cohn, 1975). Although widely occurring in vertebrate tissues, it appears to be synthesized mainly in the hypothalamus and the endocrine pancreas, in a ribosome-dependent manner via a much larger precursor molecule (Ensinck et al., 1978; Noe et al., 1979; Joseph-Bravo et al., 1980; Patzelt et al., 1980). It thus conforms to the model already established for the biosynthesis of other peptide hor-

† From the Institut für Physiologische Chemie der Universität Hamburg, Abteilung Zellbiochemie, 2000 Hamburg 20, Federal Republic of Germany. *Received July 2, 1981.* R.I. was supported by a fellowship of the Alexander von Humboldt Stiftung, Bonn, Federal Republic of Germany.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; IgG, immunoglobulin G fraction; NpII, bovine neurophysin II; anti-NpII, rabbit antibodies to bovine neurophysin II; ppAVP/NpII and pAVP/NpII, prepro and pro forms, respectively, of the arginine vasopressin/neurophysin II common precursor; SRIF, tetradecapeptide somatostatin; anti-SRIF, rabbit antibodies to synthetic somatostatin; ppSRIF and pSRIF, prepro- and prosomatostatin, respectively; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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