LIGHT INCREASES THE ION AND NON-ELECTROLYTE PERMEABILITY OF RHODOPSIN-PHOSPHOLIPID VESICLES

A. Darszon,* M. Montal* and J. Zarco

Departamento de Bioquímica, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, México 14, D.F., MEXICO

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SUMMARY.- Rhodopsin-phospholipid vesicles were formed and loaded with radioactive markers by sonication. Exposure of the vesicles to light increased the permeability to Na^+, Cs^+, Ca^{2+}, glycerol and glucose, but not to Cl^-, sucrose and inulin. These results indicate that light induces the formation of a wide permeability pathway with an apparent cut-off diameter of about 10Å.

INTRODUCTION

Photon capture by rhodopsin, a chromophore-bearing membrane protein constituent of photoreceptor cells, triggers the excitation of the visual system (1-2). The absorbed light isomerizes the chromophore, retinaldehyde, from the 11-cis to the all-trans configuration (3). As a consequence of this, the protein goes through conformational changes which, in course, lead to a modification of the photoreceptor plasma membrane permeability (2). Rhodopsin, however, is located in the intracellular discs. A major unsolved question, therefore, concerns the transduction mechanism by which excited rhodopsin molecules in the discs change the cell membrane permeability and thus generate the amplified excitatory response. Rhodopsin could achieve this light energy conversion by functioning as a light-activated ion translocator (3,4,5).

We have approached this problem by incorporating rhodopsin into bilayer membranes to evaluate the light-induced changes in ion-permeability (4,5,6). Our findings with rhodopsin incorporated into planar bilayers

*Present address: Departments of Physics and Biology, University of California, San Diego, La Jolla, California 92093

Abbreviations: ROS, rod outer segments; CTAB, cetyltrimethylammonium bromide; PB, phosphate buffer; IB, imidazole-HCl buffer; DEAE, diethylaminoethyl; EM, electron microscopy, ΔA_{500}, difference in absorbance at 500 nm between dark and bleached rhodopsin samples.

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indicate that it functions as a light-activated and voltage-sensitive channel (6). Here we report complementary data derived from rhodopsin incorporated into bilayer vesicles and show that, in agreement with the planar bilayer results, light increases the bilayer vesicle permeability to some ions and non-electrolytes in a pattern consistent with the formation of a wide permeability pathway of around 10Å in diameter.

METHODS AND MATERIALS

All procedures were carried out at 2-4°C under dim red light (Kodak series 1 filter) unless otherwise stated.

Rod outer segments (ROS) from dark adapted bovine retinas (Hormel Co.) were isolated by sucrose flotation and purified in a discontinuous sucrose gradient (7). Rhodopsin from purified ROS was extracted with 50 mM cetyltrimethylammonium bromide (CTAB) - 66 mM phosphate buffer, pH 7.0 (CTAB-PB) or with 50 mM CTAB - 10 mM imidazole - HCl buffer, pH 7.0 (CTAB-IB) (8). The concentration of the CTAB solubilized rhodopsin was measured from the difference in absorbance ΔA at 500 nm (ΔA_{500}) between dark and bleached samples, assuming an extinction coefficient of 40,000 $M^{-1}cm^{-1}$ (9) and a molecular weight of 40,000 daltons (10). Retinal-free opsin was prepared according to Hubbard et al. (10) and extracted with CTAB-PB.

Solubilized rhodopsin was incorporated into spherical bilayers as follows: soybean phospholipids, partially purified (11), were mechanically dispersed at 10 mg/ml on a vortex mixer. The dispersing solution was 0.01 M imidazole buffer pH 7.0, containing 1 mM $CaCl_2$ and was 0.1 M (10^6cpm ml^{-1}) with respect to the molecular species under permeability investigation. The Ca^{++} experiments were done in 0.1 M KCl and 1 or 2 mM $CaCl_2$ (10^6 cpm $^{45}Ca^{++}$ ml^{-1}). $^{22}Na^+$, $^{134}Cs^+$, $^{36}Cl^-$, ^{3}H-glycerol, ^{3}H-glucose, ^{3}H or ^{14}C-inulin and ^{14}C-CTAB were from Amersham/Searle Corp. Solubilized rhodopsin (0.3 ml) at 2.5 mg/ml in CTAB-PB or CTAB-IB was incubated for 1 h at room temperature (22 ± 2°C) with humid Bio Beads (Bio Rad Laboratories) (187 x weight of protein) in order to remove most detergent from the protein sample (12). After this step the Bio Beads treated rhodopsin solution was cosonicated with 2 ml of the lipid suspension for 3.5 min by immersion of the test tube in a water bath sonicator (Bransonic, Heat Systems, Inc. Plainview, N.Y., ultrasonic cleaner-power output 100 watts) (13).

Column chromatography on DEAE-cellulose was used to measure the light induced changes in permeability (14). Humid DEAE cellulose (1.5 ml) was vacuum packed in a column and equilibrated with 0.1 M, KCl containing 10 mM imidazole pH 7.0. In the Cl^- experiments the columns were equilibrated with 0.1 M potassium phosphate buffer, pH 7.0. The photostimulator consisted of a filament light-source, a heat filter, Interference Filters (Baird Atomic, narrow band) and a condensing lens. Fully bleached (500 nm illumination) and control (no illumination) sonicated vesicles (0.5 ml) were transferred to the column and introduced. The column was then washed with 4 column volumes of the salts used for equilibration, and the eluate containing radioactivity not enclosed by vesicles, was reserved for counting. The radioactivity enclosed within vesicles was measured after elution with 2 column volumes of 1%(w/v) Triton x-100-PB. Aliquots from detergent samples were used to measure rhodopsin. The retained label is expressed as the percentage of the total amount of radioactivity recovered from the DEAE column. The light induced change in permeability, expressed as a percentage, is defined as: $(D-L)/D \times 100 = \underline{\Delta P}$, where D = retained label by dark vesicles and

L = retained label by bleached vesicles. The results were analyzed using a t Student test for means. The value of p and of n (number of experiments) are indicated.

Rhodopsin incorporated into bilayer vesicles was regenerated by the following procedure: vesicle aliquots were dissolved in CTAB-PB to measure the rhodopsin concentration. The vesicles were then bleached completely and incubated in the dark for 12 h with 9-cis retinal (Sigma Chemical Co.) (a two-fold excess on a molar basis) (15). An aliquot of the incubated vesicles was dissolved in CTAB-PB and the isorhodopsin concentration measured (ΔA485) (15). The amount of regeneration of isorhodopsin is expressed as a percentage of the original rhodopsin concentration.

The size distribution of the vesicles was determined by molecular sieve chromatography on a lipid pretreated Sepharose 4B-200 column (2.5 x 50 cm) according to Huang (16).

Phospholipid phosphate was analyzed according to Dawson (17) and protein according to Lowry (18). Absorption spectra were recorded in a Cary 14 spectrophotometer (Applied Physics Corp.). Radioactivity was measured in a Packard Tri Carb Liquid Scintillation Spectrometer model 3003, utilizing detergent-based toluene liquid scintillation solution (19), or in a Nuclear Chicago well type counter.

RESULTS

The vesicles were examined by negative staining and freeze-fracture electron-microscopy (EM). The predominant population consisted of unilamellar vesicles of about 2500 Å in diameter. Their fracture faces showed a random distribution of particles of about 100 Å in diameter (20). In agreement, molecular sieve chromatography indicates that the preparation consisted mainly of one homogeneous population of vesicles, with a phospholipid to rhodopsin molecular ratio of $\sim 2.5 \times 10^3$, implying the presence of extensive regions of lipid bilayer. The vesicles contained residual detergent, equivalent to a ratio of CTAB molecules per rhodopsin of $\sim 2 \times 10^2$. Rhodopsin is not regenerable in CTAB solutions; however after detergent removal it can be regenerated (12). Under the conditions described for vesicle formation rhodopsin was regenerated with 9-cis retinal up to 56.25 ± 14.68% (n=5) (15).

The existence of sealed vesicles was established by measuring the efflux of labeled ions induced by the addition of ionophores X-537A and gramicidin (21). In the specific case of rhodopsin vesicles loaded with NaCl (0.1 M, 10^6 cpm/ml $^{22}Na^+$) gramicidin induced the leakage of 35 ± 7.07 % (n=2) of their original Na^+ content.

Exposure of rhodopsin-lipid vesicles to light increased the permeability to some ions and non-electrolytes. The difference in the release of labeled components from control and bleached vesicles expressed as ΔP is illustrated in Fig. 1. The release was measured within a standardized time

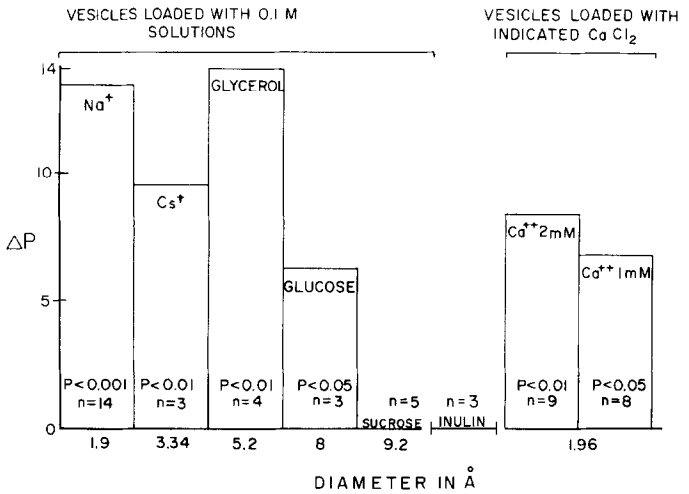

FIGURE 1. Light induced change in permeability of the vesicles (ΔP) to ions and non-electrolytes. The vesicles were prepared and loaded with the corresponding label according to materials and methods. The inulin experiments were performed in the presence of 10 mM cold inulin, and its molecular weight is 7000. Occasionally a rhodopsin preparation did not show a light response; these experiments have been included in the statistical analysis. The bars represent the mean value of ΔP (as defined in materials and methods). The numbers under each column indicate the ionic or molecular diameters taken from reference (25) or when not available, estimated from measurements on molecular models. Inside the columns appear the number of experiments, n , and the p value of a t Student test. The D values determined in these experiments (percentage of label retained by dark vesicles) were: $^{22}Na^{+}$ 2.71 ± 0.7, n=14; $^{134}Cs^{+}$ 2.42 ± 0.6, n=3; ^{3}H-Glycerol 0.45 ± 0.04, n=4; ^{3}H-Glucose 2.18 ± 0.06, n=3; ^{14}C or ^{3}H-Sucrose 2.28 ± 0.31, n=5; ^{14}C or ^{3}H-Inulin 1.86 ± 0.32, n=3; $^{45}Ca^{++}$ (1mM), 24.51 ± 2.57, n=8; $^{45}Ca^{++}$ (2mM), 27.07 ± 4.82, n=9 (mean ± one standard deviation, n = number of experiments.

of 2.5 min. while the vesicles were subjected to an ionic gradient in a column. Despite of differences in ionic radius Na^{+} and Cs^{+} ions are released to about the same extent, on the order of 10^{2} ions per bleached rhodopsin molecule. Among the non-electrolytes, the permeability of glycerol and glucose is also increased by bleaching whereas the sucrose and inulin permeabilities remain unaffected by illumination. These experiments were performed in the presence of 1 mM $CaCl_2$, which is required for optimal effects (4). Release of Ca^{++} was measured at the concentrations of 1 and 2 mM in order to avoid precipitation of the vesicles. Measurements of $^{36}Cl^{-}$ release did not show a difference between control and bleached samples. These results indicate that light induces the formation of a wide permeability pathway with a cut-off diameter in between that of glucose and sucrose, i.e. about 10 Å (22).

TABLE I

WAVELENGTH DEPENDENCE OF THE LIGHT-INDUCED PERMEABILITY RESPONSE OF RHODOPSIN VESICLES TO Na^+ AND Ca^{++}

Light Wavelength nm	Extent of ion release from vesicles by light $Na^+ \times 10^{16}$	n	$Ca^{++} \times 10^{15}$	n
600 ± 10.63	1.15 ± 1.63	2	0.73 ± 1.03	2
540 ± 11.25	3.42 ± 0.7	3	4.2 ± 0.39	2
500 ± 9.38	4.99 ± 0.16	3	6.37 ± 2.84	2
440 ± 8.75	2.64 ± 0.61	3	1.62 ± 0.15	2
400 ± 9.38	2.45 ± 0.12	2	0.94 ± 0.57	2

The vesicles (formed as in methods and materials) were exposed to equivalent integral light intensities at the indicated wavelengths and introduced into the DEAE column within 10 min after beginning of the exposure. The results represent the mean value ± one standard deviation of the difference between dark and light exposed samples expressed as the differential release of the ion; n , indicates the number of experiments. Narrow band interference filters were used. Their bandwidth at 50% transmittance is indicated. Temperature, 2-4°C.

To show that rhodopsin is responsible for the observed permeability effects, the wavelength dependence of the light response for Ca^{++} and Na^+ was studied (4). This was accomplished by exposing parallel samples of rhodopsin vesicles to a constant number of photons and comparing their ion content with respect to a dark control. In these experiments, therefore, the only variable was the wavelength of the light applied to the sample. For the experiment illustrated in Table I, the light intensity was chosen so as to bleach 67% ± 16 (n=5) of the rhodopsin present in the vesicles. The results show that the efflux peak occurred at the rhodopsin absorption maximum of 500 nm and fell to both sides of the spectrum, attaining the lowest values at 400 and 600 nm. These values fit the absorption spectrum of rhodopsin within the experimental error.

In order to better define which of the photo-intermediates is capable of changing the permeability, the Na^+-content of control and bleached

rhodopsin vesicles and of retinal-free opsin vesicles was compared. All the experiments were carried out at temperatures between 2-4°C, so that the product of bleaching during the time of the experiments consists mainly of metarhodopsin II, a covalent complex of opsin and all-trans retinal (2). This was done to avoid the release of free retinal which is known to increase the K^+ permeability of phosphotidylethanolamine vesicles (23). The results demonstrate that the Na^+ content (percent retained) of opsin vesicles (1.53%) is lower than that of the unbleached rhodopsin vesicles (2.15%) but comparable to that of bleached rhodopsin vesicles (1.85%). Therefore, opsin, as well as metarhodopsin II, are capable of altering the bilayer permeability.

DISCUSSION

Light increases the permeability of rhodopsin-lipid vesicles to the cations Na^+, Cs^+ and Ca^{++} and to the non-electrolytes glycerol and glucose. The wavelength-dependence of this phenomenon together with a set of control experiments that exclude the involvement of retinal and detergent (4), establish that the light response can be accounted for by rhodopsin bleaching. The permeation pathway appears to be poorly selective since all cationic species tested pass through with an apparent equivalent efficiency. However, no light response was observed with vesicles loaded with Cl^-. This could be attributed to the exclusion of anions from the pathway. Among the non-electrolytes tested the largest molecules, sucrose and inulin, do not permeate through the light-induced pathway. The evidence is, therefore, consistent with the light-induced formation of a wide permeability pathway with a diameter of about 10 Å.

Alternative interpretations of the results are conceivable, such as: (a) light-induced vesicle fusion; (b) light-induced changes in binding of labelled species to rhodopsin; (c) different affinities of dark and bleached vesicles to the DEAE columns. The first and last alternatives are inconsistent with the fact that the sucrose and inulin permeabilities are unmodified by light exposure. The second possibility is rendered unlikely by the result of the light-induced release of glucose and glycerol, two entities with poor binding to macromolecules. Therefore, our favored interpretation is that light excitation of rhodopsin leads to the formation of a transmembrane channel.

We have obtained additional evidence in favor of this interpretation by incorporating rhodopsin into planar bilayer membranes. In this system, exposure to light increases the membrane conductance (5,6). The conductance fluctuates in discrete steps similar to those induced in lipid bilayers by molecules that form transmembrane channels (21). From the magnitude of the

conductance fluctuations, the diameter of the channel can be estimated to be 10-15 Å (6). Recent hydrogen-tritium exchange measurements in rod disc membrane suspensions are also consistent with the formation of a hydrophilic channel of about 10-15Å in diameter (24).

The channel in planar bilayers is cation selective and voltage sensitive: it is open in the vicinity of zero voltage and closes with applied voltages of either polarity (6). The small light-induced change in vesicle permeability, $\Delta P_{light} \sim 14\%$, for Na^+ in comparison to that induced by the voltage insensitive channel gramicidin, $\Delta P_{gramicidin} \sim 35\%$, could be attributed to the voltage-sensitivity of the rhodopsin channel in the bilayer vesicles: during the experiments the vesicles are subjected to an ionic gradient across the membrane; consequently, the light-activated opening of the channel should be followed by a diffusion potential eventually sufficient to close the channel (6).

On the basis of these findings with vesicles and those obtained with planar bilayer (6) a model of vertebrate visual phototransduction that considers Ca^{2+} as the transmitter (2) can be proposed, as follows. In the dark, the channel is closed and the Ca^{2+}-dependent adenosine triphosphatase would pump Ca^{2+} into the discs; on bleaching, the channel would form and allow Ca^{2+} to diffuse down its concentration gradient. The efflux of Ca^{2+} would change the disc membrane potential to the value at which the channel closes. The channel closure and the photoregeneration of rhodopsin, as well as the activity of the pump, restore the membrane to the conditions prevalent in the dark (6).

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