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ASSOCIATION OF BACTERIORHODOPSIN WITH LIPID-IMPREGNATED FILTERS

EVIDENCE FOR FUSION OF BACTERIORHODOPSIN-CONTAINING VESICLES WITH THE LIPID PHASE OF THE FILTER

M.C. BLOK and K. VAN DAM

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Plantage Muidergracht 12, 1018 TV Amsterdam (The Netherlands)

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Summary

Bacteriorhodopsin vesicles were associated with cellulose-nitrate filters impregnated with a solution of phospholipids in hexadecane. The generation of (photo)potentials upon illumination of the filter was studied in the absence and presence of ionophores, phospholipase A_2, EDTA or polyene antibiotics.

From these experiments the following conclusions are drawn.

1. Upon illumination of the filter, bacteriorhodopsin pumps protons into aqueous compartments located in the filter.

2. These aqueous compartments possibly do not originate from the compartments enclosed by the bacteriorhodopsin vesicles. Evidence is obtained that aqueous compartments are present in the surface layers of the lipid-impregnated filters.

3. The results are explained most easily by a mechanism, whereby fusion occurs between the vesicles and the lipids of the filter.

Introduction

An approach to the study of the properties and mechanism of action of transport proteins is the reconstitution of these proteins into model membrane systems. Besides the liposomal system, artificial planar membranes have been used for this purpose [1]. Successful incorporation of membrane proteins in the latter system has been achieved in several ways, e.g. by dispersion into the alkane solution used to prepare the membrane [2] or simply by addition to the

Abbreviations: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone; ACMA, 9-amino-6-chloro-2-methoxyacridine.

aqueous phase on one side of the membrane [3–5]. In another approach vesicles, e.g. natural membrane vesicles prepared from sarcoplasmic reticulum [6] or gastric mucosa [7], are associated with planar membranes in the presence of Ca^{2+}.

Apart from these studies with membrane proteins, the interaction of lipid vesicles with planar membranes has been used as a model for fusion of vesicles with biological membranes [8—11].

The light-sensitive protein bacteriorhodopsin has been incorporated into planar membranes by all the methods described above [12—18]. This chromoprotein is the only protein present in specialized regions of the plasma membrane of some halophilic bacteria (e.g. *Halobacterium halobium*), the so-called purple membrane [19]. It has been concluded from both in vivo and in vitro studies that bacteriorhodopsin is able to convert the energy of absorbed light into that of an electrochemical gradient of protons [17, 20—24]. Bacteriorhodopsin can be readily incorporated into lipid vesicles [21,23] in such a way that a net proton movement can be observed upon illumination. Association of these vesicles with planar membranes is accomplished by addition of divalent ions, e.g. Ca^{2+}, to the bathing solution. The stability of the classical Mueller-Rudin films has been improved by increasing the concentration of phospholipids in the decane solution [13,17], by incorporating polymers such as polystyrene in the membrane-forming solution [16] or by using lipid-impregnated, cellulose-nitrate filters [25—27]. All of these methods yield model systems whose thickness is considerably increased, compared to that of black lipid membranes. As far as the nature of the association of bacteriorhodopsin vesicles with these model systems is concerned, no clear-cut description has been given. Several investigators [13,17,18] have put forward models in which bacteriorhodopsin pumps protons primarily into an aqueous compartment located between the two bulk aqueous compartments. It has been stated that the vesicles retain their structure during association with planar membranes [17]. However, the terms "adhesion" and "fusion" are used arbitrarily to describe the nature of vesicle association [13,17]. The present study reports results concerning the question of how bacteriorhodopsin vesicles are associated with lipid-impregnated filters.

Materials and Methods

Purple membranes [28] and soya-bean phospholipids [29] were isolated according to procedures described elsewhere. Egg phosphatidylcholine was a gift of Dr. J. de Gier. Cardiolipin was obtained from Sigma Chemical Co. Ltd. (St. Louis, Mo., U.S.A.). Octadecylamine was obtained from Merck (Darmstadt, F.R.G.).

Phospholipid vesicles containing bacteriorhodopsin were routinely prepared in 150 mM KCl as described previously [27]. Sartorious filters (cellulose nitrate, pore size 0.15 μm, 0.15 mm thick) were impregnated with soya-bean phospholipids dissolved in hexadecane (75 or 150 mg lipid/ml). Excess lipid solution was removed and the filter clamped between two 20-ml Teflon vessels, each containing a circular aperture of 12 mm diameter. Association of the vesicles with the filter was carried out as follows. The suspension of bacteriorho-

dopsin vesicles (10 mg of soya-bean phospholipid/ml) was mixed with an equal volume of 100 mM $CaCl_2$. Subsequently, 0.25 ml of this mixture was pipetted onto the upper side of the filter, which had been placed in a horizontal position by tilting the holder. After 15 min the vesicles that were not associated with the filter were removed by withdrawing the aqueous suspension. The filter was placed in its normal (vertical) position and the compartments on the two sides of the filter were filled with medium (75 mM KCl, 50 mM $CaCl_2$, unless otherwise stated). The solutions in both compartments were stirred gently by means of magnetic stirrers.

Each illumination was preceded by a 15 min equilibration period in the dark. Illumination of the filter (from one side) was achieved using a 250 W projector lamp and fiber optics. During a 1 min illumination period the photopotential reached its maximal value. When the effect of a certain substance on the photopotential was studied, it was added 1 min after finishing illumination as described above, followed by another equilibration period of 15 min. Subsequently, the photopotential generated during a second illumination period of 1 min was monitored. Usually, the effect of an addition is presented as the relative change of the potential: $(\psi_2 - \psi_1/\psi_1) \times 100\%$, where ψ_1 and ψ_2 are the maximal photopotentials generated during illumination before and after addition of the compound to be tested. Potentials were measured with an electric circuit described previously [26].

Measurement of the H^+ uptake during illumination of a suspension of bacteriorhodopsin vesicles was done as described elsewhere [30]. The light-driven fluorescence quenching of 9-amino-6-chloro-2-methoxyacridine (ACMA) by bacteriorhodopsin vesicles was measured using an apparatus built by Dr. Fiolet [31].

Purified phospholipase A_2 from *Naja naja* was a generous gift of Dr. R.F.A. Zwaal. Filipin was kindly supplied by the Upjohn Chem. Co. (Kalamazoo, Mich., U.S.A.) and amphotericin B by the Squibb Institute for Medical Research (New Brunswick, N.J., U.S.A.). ACMA was synthesized by Dr. R. Kraayenhof [32]. Gramicidin was obtained from California Found., Biochemical Research. Valinomycin and nigericin were gifts from Eli Lilly and Co. Carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) was a gift from Dr. P.G. Heytler. All other reagents were obtained commercially and were of analytical reagent grade. Stock solutions of amphotericin B in dimethylsulfoxide (20 mM) and of other polyene antibiotics in dimethylformamide were prepared on the day of use.

Results

(I) The effects of ionophores

It has been shown previously that the (photo)potential, which is generated by bacteriorhodopsin associated with a lipid-impregnated filter, is influenced by the addition of ionophores [26]. Table I shows that in most cases ionophores are effective only if they are added to the aqueous compartment on that side of the filter where the bacteriorhodopsin vesicles have been associated (which is referred to as compartment I). Under this condition, the dissipation of the photopotential depends on the concentration of the ionophore and

TABLE I

CHANGE OF THE PHOTOPOTENTIAL CAUSED BY THE ADDITION OF IONOPHORES ON DIFFERENT SIDES OF THE FILTER

The experiments were carried out as described in Materials and Methods. Compartment I is at the bacteriorhodopsin-containing side of the filter; Compartment II is at the opposite side. Addition of equivalent amounts of ethanol, in which the ionophores were dissolved, had no effect on the photopotential.

Ionophore	Addition to compartment	Photopotential (mV)		Percent change of the potential
		Before addition	After addition	
Control (no additions)	—	164	160	−2
Gramicidin (1 ng/ml)	I	129	84	−35
	II	149	147	−1
Valinomycin (0.5 μg/ml)	I	152	101	−34
	II	143	140	−2
Nigericin (1.0 μg/ml)	I	159	179	+13
	II	154	151	−2
FCCP ($4 \cdot 10^{-6}$ M)	I	146	98	−33
	II	138	126	−9

reaches approx. 90% of its maximal effect in 15 min (data not shown). Addition of the ionophores to the opposite aqueous compartment (compartment II) had a significant effect only in the case of FCCP (Table I), and this is enhanced after prolonged incubation (not shown). On the other hand, even 60 min after addition of valinomycin to compartment II no significant decrease could be observed. Moreover, lack of effect of nigericin and valinomycin on the photopotential did not depend on the ionic composition of the medium in compartment II. To achieve an effect on the photopotential, compartment I should contain alkali ions that can be transported by these ionophores (data not shown).

The addition of ionophores often resulted in a change in electric potential across the filter in the dark. Therefore, we studied in some more detail what happens when ionophores are added to lipid-impregnated filters with no associated bacteriorhodopsin vesicles. Fig. 1A shows that under these conditions addition of valinomycin to one compartment results in a change of the potential. This potential decays very slowly. Introduction of valinomycin into the second compartment, which contains the same medium, results in a change of the potential of the same magnitude, but in the opposite direction. Even at a relatively low valinomycin concentration the change of the potential is independent of the amount of ionophore added (Fig. 1B), whereas the rate of change does depend on the antibiotic concentration. This indicates that this phenomenon is not due to a stoichiometric binding of potassium plus valinomycin by the filter. The magnitude of the change depends on the concentration of K^+ in the compartment to which the antibiotic is added (Fig. 1C). The data suggest a proportionality with the logarithm of the potassium concentration, suggesting that the potential changes may be due to the generation of diffusion potentials. In analogous experiments we found that also gramicidin induced such a change of the potential. This change, however, was relatively small. After the potential had reached a plateau, a further change of the potential

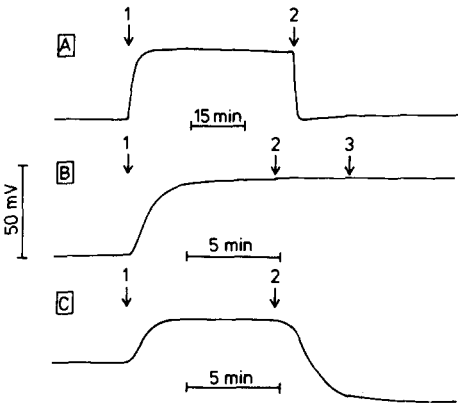


Fig. 1. Changes of potential induced by the addition of valinomycin to filters impregnated with a hexadecane solution of soya-bean phospholipids. A filter was impregnated with a solution of soya-bean phospholipids in hexadecane (75 mg phospholipid/ml) and clamped between two Teflon vessels. After filling both compartments with medium the system was equilibrated until the potential was constant with time. In Expts. A and B, the medium on both sides of the filter was 75 mM KCl, 50 mM $CaCl_2$; the media in Expt. C are given below. In Expt. A, valinomycin was added to compartment I (arrow 1) and compartment II (arrow 2) to a final concentration of 0.5 μg/ml. In Expt. B the following amounts of valinomycin were added to 20 ml medium contained in compartment I: arrow 1, 2 μg; arrows 2 and 3, 4 μg per addition. In Expt. C 4 μg valinomycin was added to compartment I that contained 7.5 mM KCl, 50 mM $CaCl_2$, 135 mM sucrose (arrow 1), and to compartment II, containing 75 mM KCl, 50 mM $CaCl_2$ (arrow 2).

could be induced by the addition of valinomycin to the same compartment. On the other hand, when valinomycin was added first, no effect was found after the subsequent addition of gramicidin. Finally, the concentration of the phospholipids in the hexadecane phase was found to be of critical importance. At a concentration of 5 mg soya-bean phospholipids per ml of hexadecane no potential change was observed upon addition of valinomycin. The effect appeared upon increasing the phospholipid concentration.

(II) The effects of phospholipase A_2

Before testing the effects of phospholipase A_2 on the generation of photopotentials by bacteriorhodopsin in the filter system, a control experiment was done to show that the hydrolysis of phospholipids damages vesicles in suspension. As a monitor of damage we used the capacity of the vesicles to accumulate protons during illumination. Since acid equivalents are liberated during phospholipid hydrolysis a buffered medium had to be used. This makes it impossible to measure the light-induced proton uptake by bacteriorhodopsin vesicles by using a pH electrode. Therefore, an indirect, qualitative method was used, namely the light-driven quenching of ACMA fluorescence [32]. Before the addition of phospholipase A_2 a significant quenching is observed during illumination of the vesicles (Fig. 2A). In contrast, 15 min after addition of the enzyme the only response observed upon illumination is an artifact, which is found also during illumination of a suspension of vesicles in the absence of ACMA (Fig. 2A). Since phospholipase A_2 is activated by Ca^{2+} [33], a control experiment was done in the presence of excess EDTA to show that the enzyme

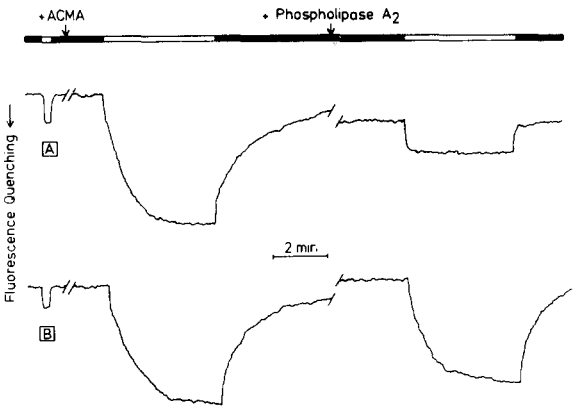


Fig. 2. The effect of phospholipase A_2 on the light-driven quenching of ACMA fluorescence by bacteriorhodopsin vesicles. After addition of 2.4 ml medium and bacteriorhodopsin vesicles to the cuvette and equilibration of the system for approx. 10 min the suspension was illuminated in order to determine the size of the light artifact. Light from a 250 W projector lamp was used in these experiments and passed through a filter that cuts off light of wavelengths less than 490 nm. Subsequently, ACMA was added to a final concentration of 8.3 μM. After a dark period of 15 min the light-dependent quenching of the fluorescence was measured by illuminating the vesicles for 4 min. A dark period of 4 min followed. Then 1.0 I.U. of phospholipase A_2 was added followed by incubation in the dark for 15 min. The effect of phospholipase on the quenching of ACMA fluorescence was measured during a second cycle of illumination, identical to the first one. In both experiments, bacteriorhodopsin vesicles were present in a final concentration of 0.83 mg soya-bean phospholipid/ml. The vesicles had a protein to phospholipid ratio of 1 : 20, w/w. ACMA was excited with light of 410 nm; emitted light was measured at 490 nm. In Expt. A the medium consisted of 50 mM Tris-HCl (pH 7.5), 10 mM $CaCl_2$ and 87.5 mM KCl; in Expt. B of 50 mM Tris-HCl (pH 7.5), 5 mM EDTA and 94 mM KCl.

itself has no significant effect on the light-dependent fluorescence quenching (Fig. 2B).

Fig. 3 shows that the addition of phospholipase A_2 at the bacteriorhodopsin-containing side of the filter results in a decrease of the potential that is generated upon illumination in this system. However, the photopotential was decreased only partially, and reached a plateau after prolonged incubation. Table II shows that phospholipase A_2 only significantly decreases the photopotential if it is added at the bacteriorhodopsin-containing side of the filter in the presence of Ca^{2+}. It can be seen also that the photopotential decays more rapidly after a second illumination of the filter. However, this decay is accelerated significantly under the condition that phospholipase A_2 has an effect on the magnitude of the photopotential.

In a previous report it was described that divalent ions, e.g. Ca^{2+}, are essential for the association of bacteriorhodopsin vesicles with lipid-impregnated filters [27]. Table II shows that removal of Ca^{2+} by addition of EDTA gave only a slightly more pronounced decrease of the photopotential. This difference did not increase on prolonged incubation. In contrast to this, the Ca^{2+}-induced turbidity of a suspension of bacteriorhodopsin vesicles was decreased by more than 85% if the Ca^{2+} concentration was lowered from 50 to 2 mM and if the suspension was mixed for 60 s (not shown), indicating that the vesicle aggregation is largely reversible.

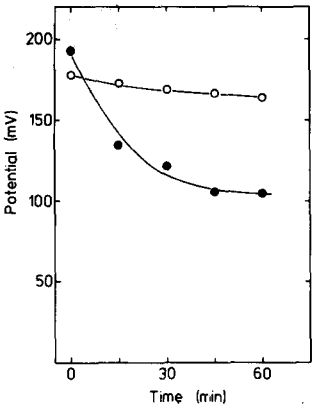


Fig. 3. The effect of phospholipase A_2 from *Naja naja* on the photopotential generated by bacteriorhodopsin. Bacteriorhodopsin vesicles were associated with a lipid-impregnated filter as described in Materials and Methods. After filling both compartments with 15 mM Tris-HCl (pH 7.5), 67.5 mM KCl and 45 mM $CaCl_2$, and equilibrating for 10 min, the photopotential was determined during an illumination period of 1 min (= zero time). Immediately after measuring the photopotential, phospholipase A_2 (5.3 I.U. per 20 ml) was added to the compartment at the bacteriorhodopsin-containing side of the filter. The effect on the photopotential was measured various times after addition (●——●). ○——○, control experiment without phospholipase.

(III) The effects of polyene antibiotics

Polyene antibiotics, e.g. amphotericin B, are active only in membranes that contain sterols [34]. The interaction of the former compounds with cholesterol, present in biological membranes or in liposomes, has been investigated thoroughly [35,36]. Amphotericin B enhances the permeability of cellular membranes [34,37], liposomes [34,37] and black lipid membranes [38,39].

TABLE II

THE EFFECT OF PHOSPHOLIPASE A_2 FROM *NAJA NAJA* ON THE PHOTOPOTENTIAL GENERATED BY BACTERIORHODOPSIN

The experiment was carried out as described in Materials and Methods. After measuring the photopotential (vertical column indicated by I) phospholipase A_2 was added and its effect was measured after 15 min by again measuring the photopotential (vertical column indicated by II).

Medium	Phospholipase present	Photopotential (mV)		Percent decrease of potential	Percent increase of the rate of decay **
		I	II		
Medium A * (plus Ca^{2+})	—	141	131	7	45
	+	152	120	21	139
	+ ***	142	139	2	25
Medium B * (plus EDTA)	—	125	111	11	32
	+	119	106	11	26

* Medium A: 15 mM Tris-HCl, 45 mM $CaCl_2$, 67.5 mM KCl, pH 7.5; Medium B: 15 mM Tris-HCl, 10 mM EDTA, 120 mM KCl, pH 7.5.

** Defined as $[\{1/t_{1/2}\ (+\ PLA_2) - 1/t_{1/2}\ (-PLA_2)\}/1/t_{1/2}\ (-PLA_2)] \times 100\%$; PLA_2, phospholipase A_2; $t_{1/2}$ is the half-time of the decay of the photopotential.

*** Phospholipase was not added at the bacteriorhodopsin-containing side of the filter as in the other cases, but to the opposite compartment.

Substantial evidence exists to support the hypothesis that the amphotericin B-induced permeability is due to the formation of conducting channels across the membrane [38,40]. These pores are composed of cylindrical arrangements of amphotericin B-cholesterol complexes. In black lipid membranes conducting channels are formed by apposition of two half pores [39]. In liposomes a half pore can span the lipid bilayer [41].

In bacteriorhodopsin vesicles that contain cholesterol the extent of light-dependent proton uptake is decreased by amphotericin B in a concentration-dependent way; on the other hand, amphotericin B had no effect on vesicles without cholesterol (data not shown). In Fig. 4 it is shown that amphotericin B caused a decrease of the photopotential, provided that cholesterol-containing vesicles were associated with lipid-impregnated filters. The effect on the photopotential increased with increasing antibiotic concentration. In another experiment the dependence of amphotericin B action on the cholesterol content of the bacteriorhodopsin vesicles was examined. These vesicles were associated to filters that did not contain cholesterol. Fig. 5 shows how the effect of amphotericin B increased with increasing cholesterol content of the vesicles. As a control we tested the effect of addition of solvent, but this gave no significant decrease of the photopotential. Fig. 6 shows the results of the reverse experiment in which bacteriorhodopsin vesicles without cholesterol were associated with filters with variable ratios of cholesterol/phospholipid in the hexadecane

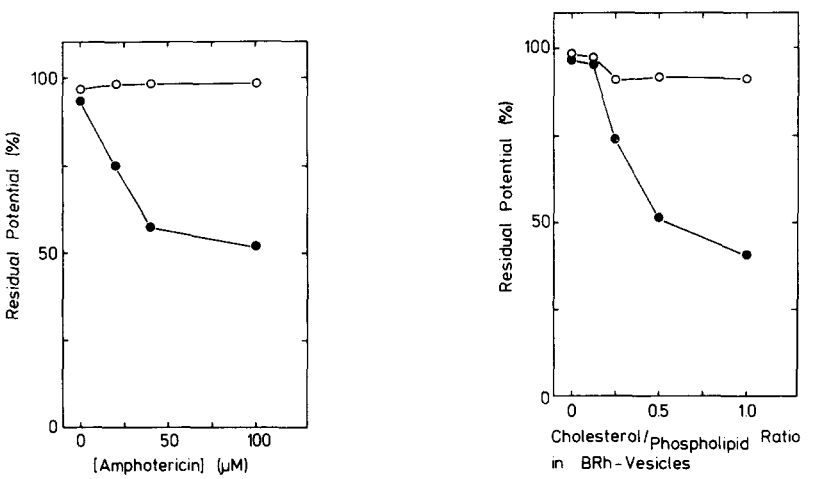
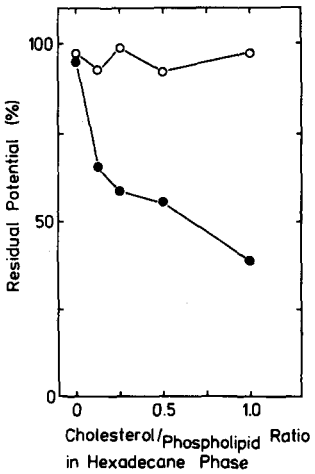


Fig. 4. Decrease of the photopotential generated by bacteriorhodopsin as a function of the concentration of amphotericin B. The experiment was carried out as described in Materials and Methods, using separate filters for each concentration of antibiotic tested. To the lipid-impregnated filters bacteriorhodopsin vesicles without cholesterol (○———○) or with a molar ratio of cholesterol to soya-bean phospholipid of 0.5 (●———●) were associated. In both cases the protein/soya-bean phospholipid ratio of the vesicles was 1 : 20, w/w.

Fig. 5. Decrease of the photopotential induced by amphotericin B. Bacteriorhodopsin (BRh) vesicles with varying cholesterol to soya-bean phospholipid ratio were associated with lipid-impregnated filters without cholesterol. The experiment was done as described in Materials and Methods. In all cases, however, the protein/soya-bean phospholipid ratio of the vesicles was 1 : 20, w/w. ●———●, plus amphotericin B (final concentration: 0.1 mM); ○———○, control experiment, in which the effect of dimethylsulfoxide was tested.



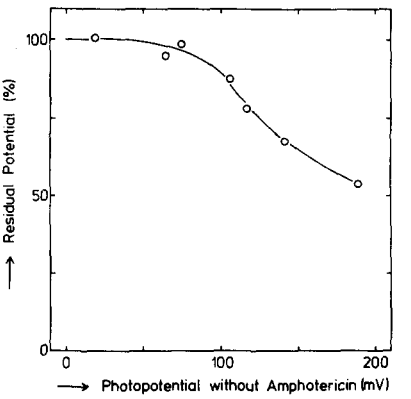


Fig. 6. Decrease of the photopotential induced by amphotericin B. Bacteriorhodopsin vesicles without cholesterol were associated to lipid-impregnated filters containing a hexadecane phase with varying molar ratio of cholesterol to soya-bean phospholipids. In all experiments the concentration of soya-bean phospholipids in hexadecane was 37.5 mg/ml. The experiment was carried out as described in Materials and Methods, using bacteriorhodopsin vesicles without cholesterol. ●———●, plus amphotericin B (final concentration, 0.1 mM); ○———○, plus dimethylsulfoxide.

Fig. 7. Amphotericin B-induced decrease of the photopotential after associating different amounts of bacteriorhodopsin vesicles with cholesterol to lipid-impregnated filters without cholesterol. The experiment was carried out as described in Materials and Methods. Variation of the amount of bacteriorhodopsin vesicles (cholesterol/soya-bean phospholipid ratio = 0.5, mol/mol; protein/soya-bean phospholipid ratio = 1 : 20, w/w) associated with the filter was achieved by varying the vesicle concentration and the incubation time during the association step. Amphotericin B was added to the vesicle side of the filter to a final concentration of 0.05 mM.

phase. Also in this case amphotericin B gave a decrease of the photopotential which increased with increasing cholesterol/phospholipid ratio. Qualitatively similar results as presented in Figs. 4—6 were obtained with another polyene antibiotic, filipin, except that in this case there was a cholesterol-independent effect at higher antibiotic concentrations (data not shown).

In Fig. 7 the results are presented of an experiment in which we examined the possibility that the association of varying amounts of cholesterol-containing vesicles with a filter without cholesterol may have an effect on the sensitivity of the photopotential to amphotericin B. As a measure of the number of bacteriorhodopsin vesicles associated with the filter we used the photopotential generated before the addition of the antibiotic [27]. Amphotericin B was found to have no effect if small amounts of vesicles were associated. When the number of associated vesicles exceeded a threshold, the effect of amphotericin B increased with the number of vesicles. As a control, we tested the effect of valinomycin and FCCP on the photopotential, and found that the relative decrease of the photopotential was constant and independent of the number of associated bacteriorhodopsin vesicles (data not shown).

In the context of this study, in which we intend to discover whether vesicles adhere to or fuse with lipid-impregnated filters, we must consider the possibility of an exchange of cholesterol. Therefore, bacteriorhodopsin vesicles with-

out cholesterol were incubated together with pure lipid vesicles with a cholesterol/phospholipid molar ratio of 0.50. Subsequently, we investigated whether or not the extent of the light-dependent H^+ uptake by the bacteriorhodopsin vesicles became sensitive to the addition of amphotericin B. After incubating the two types of vesicles together for 1 h this was not the case. This was found both in 150 mM KCl, and in 75 mM KCl, 50 mM $CaCl_2$. In the latter medium the vesicles became highly aggregated. On the other hand, co-sonication of the two types of vesicles yielded bacteriorhodopsin vesicles in which the proton uptake was reduced by approx. 30% after addition of amphotericin B. Since alkanes promote fusion of liposomes [9], we could not study the exchange of cholesterol after introduction of hexadecane into the incubation system.

(IV) Association of vesicles by two different methods and the effects of ionophores

It has been demonstrated [14] that the negatively charged purple membranes can be associated with positively charged lipid membranes in the absence of divalent cations. We found that also in the absence of Ca^{2+} bacteriorhodopsin vesicles can be associated with filters impregnated with a hexadecane solution containing 50 mg egg lecithin and 2 mg octadecylamine per ml. In Table III, the photopotentials generated in this filter system are compared to those in our usual filter system. The maximal photopotentials obtained were approx. 20—40% lower with positively charged filters (data obtained in the absence of gramicidin). In addition, it was found that the ionophores valinomycin, nigericin and FCCP had comparable effects in both systems (data not shown). Gramicidin, however, produced effects which were significantly different. To obtain a decrease of the photopotential of approx. 20%, a 10-fold higher concentration of gramicidin had to be used in the case of a filter impregnated with lecithin and octadecylamine compared with the system used normally (Table III).

TABLE III

COMPARISON OF THE EFFECT OF GRAMICIDIN ON THE PHOTOPOTENTIAL AFTER ASSOCIATION OF BACTERIORHODOPSIN VESICLES TO FILTERS CONTAINING DIFFERENT LIPIDS

The experiment was carried out as described in Materials and Methods.

Addition	Concentration (ng/ml)	Lipid in filter *	Photopotential (mV)		Percent decrease of potential
			Before addition	After addition	
None	—	egg PC + OA	98	97	—1
		SBPL	137	137	0
Gramicidin	2.5	egg PC + OA	94	87	—7
		SBPL	136	103	—24
	25	egg PC + OA	94	78	—17
		SBPL	136	52	—62

* Filters were impregnated with a hexadexane solution of egg phosphatidylcholine (egg PC) and octadecylamine (OA) (20 and 1 mg/ml, respectively) or with a hexadecane solution of soya-bean phospholipids (SBPL) (75 mg/ml).

TABLE IV

COMPARISON OF THE DIRECTION OF NET H^+ MOVEMENT AND THE DIRECTION OF THE PHOTOPOTENTIAL

Bacteriorhodopsin vesicles were prepared as follows: A solution of cardiolipin was evaporated to dryness. After dispersing the lipid in 150 mM KCl (final lipid concentration 10 mg/ml) the suspension was sonicated 60 times 15 s as described in Materials and Methods. Subsequently 0.5 ml vesicle suspension was mixed with 0.30 ml purple membrane suspension (2.0 mg protein) in water and sonicated for six times 15 s (Expt. A). In Expt. B the pH was adjusted to pH 2.5 after mixing vesicles and purple membranes. Subsequently, the mixture was sonicated for two times 15 s and the pH readjusted to pH 6.0.

Expt.	Bacteriorhodopsin vesicles prepared at	Light-driven H^+ movement *		Photopotential **	
		Direction	Extent	Direction	Size (mV)
A	pH 6.0	Accumulation	+16	—	34
B	pH 2.5	Extrusion	−35	—	15

* Measurement of the net H^+ movement during illumination of a suspension of bacteriorhodopsin vesicles was done as described [30]. The extent is given in nequiv. H^+/mg protein.

** Bacteriorhodopsin vesicles (3.1 mg cardiolipin/ml) were associated for 1 h with filters impregnated with a solution of egg lecithin (40 mg/ml) and octadecylamine (2 mg/ml) in hexadecane. After removal of non-associated bacteriorhodopsin vesicles both compartments were filled with 20 ml 150 mM KCl, and the photopotential was determined after equilibrating for 15 min as described in Materials and Methods. The direction of the potential generated during illumination of the filter is indicated as the potential measured by the electrode in the compartment on the vesicle-side of the filter minus that measured by the electrode in the opposite compartment.

(V) Bacteriorhodopsin vesicles with opposite net direction of H^+ movement

By varying the pH of the medium during preparation of bacteriorhodopsin vesicles according to the sonication procedure, vesicles can be obtained with opposite net direction of H^+ movement during illumination [42,43]. These vesicles, with cardiolipin as exogenous lipid, cannot be associated with normal filters in the presence of Ca^{2+}. Addition of Ca^{2+} to these vesicles causes fusion and a loss of the ability to extrude protons. Therefore, we associated these vesicles with filters that were positively charged. The experiment of Table III indicates that the behaviour of bacteriorhodopsin per se is not altered by associating vesicles to the filter when using this method. A suspension of vesicles prepared at pH 2.5 showed a net proton extrusion, whereas a suspension prepared at pH 6.0 showed a net proton uptake (Table IV). The extents of H^+ movement are of the same order of magnitude as those reported by Happe et al. [42]. Subsequently, we investigated whether the sign of the photopotential, generated by bacteriorhodopsin in the filter system, depended on the direction of net proton movement in the vesicles before association. There was no such dependence (Table IV), although the photopotentials were relatively small with these preparations. This may be due, however, to the lipids used in this experiment.

Discussion

(A) Generation of photopotentials: localization of the proton-accepting compartment

The photopotential generated by bacteriorhodopsin is dissipated by ionophores (Table I), phospholipase A_2 (Table II) and polyene antibiotics (data

not shown) only if these compounds are added at the bacteriorhodopsin-containing side of the filter. Addition of these compounds to either compartment did not significantly decrease the resistance of the lipid-impregnated filter (not shown). The only exception to this is FCCP, which decreased the photopotential after addition to compartment II and increased the conductance of the filter at least 10-fold (see ref. 26). We conclude that (with the possible exception of FCCP) the reduction of the photopotential, induced by the above additions, is not due to an enhanced back leakage of ions from compartment II to compartment I. To explain the decrease of the photopotential we postulate the presence of a third aqueous compartment (compartment III), which is located between compartments I and II. Upon illumination, bacteriorhodopsin pumps protons across the layer separating compartment I from this third compartment. The decrease of the photopotential induced by the above additions is ascribed to an enhancement of the permeability of this layer. Gramicidin and amphotericin B are believed to act as pore formers [38,40,44]. Since both give a decrease of the photopotential, the layer between compartments I and III must be very thin. Probably it is a bilayer-like structure.

Compartments analogous to compartment III are present in the models put forward by several other investigators [13,17,18]. In these models, this aqueous compartment is identical to the original compartment enclosed by the vesicles, which are assumed to retain their structure during association with planar membranes [13,17]. We observed, however, that the addition of valinomycin (Fig. 1) and gramicidin to lipid-impregnated filters, without any associated vesicle, resulted in a change of the electric potential. This is most likely a diffusion potential. The size of this change depends on the lipid concentration in the hexadecane phase. These results strongly suggest that within the hexadecane phase, aqueous compartments are already induced by the lipids dissolved in this phase. Some of these compartments are separated from compartment I by a bilayer-like structure, since gramicidin, which can only span a relatively thin layer, also gives a change in electric potential. In this respect it is of interest that similar aqueous compartments probably are present in planar membrane systems, prepared from a dispersion of purple membranes and azolectin in decane [17]. We conclude that compartment III is not necessarily formed by the compartment originally enclosed by the bacteriorhodopsin vesicles.

(B) Do bacteriorhodopsin vesicles adhere to or fuse with the filter?

Before discussing in more detail the outcome of experiments designed to answer this question it is useful to clarify our terminology. In our description adhesion is visualised as an association of the vesicles with the filter devoid of any continuity between these two structures. The vesicles are bound to the filter by electrostatic interactions which can be nullified, implying that the association is reversible. When the phospholipids of the lipid bilayer of the vesicle and those of the hexadecane phase of the filter form a continuous layer we consider fusion to have occurred.

Most experiments reported here are not easily explained by an adhesion mechanism. Firstly, phospholipase A_2 gave only a partial reduction of the photopotential (Fig. 3). In analogy to the effect of phospholipase A_2 on bac-

teriorhodopsin vesicles in suspension (Fig. 2), is was expected that the photopotential would disappear completely if the vesicles would adhere to the filter. The possibility that a proportion of the adhering vesicles is inaccessible for the enzyme can be excluded for several reasons. The strongest argument in this respect is that one would not expect a drastic acceleration of the decay of the remaining photopotential after phospholipase treatment (Table II) if this potential was generated by vesicles that cannot be attacked by the enzyme.

Secondly, in another series of experiments, it was found that the photopotential was reduced by the addition of amphotericin B only after introduction of cholesterol. However, cholesterol can be introduced either by using bacteriorhodopsin vesicles that contain cholesterol (Fig. 5) or by dissolving it in the hexadecane solution (Fig. 6). In either case the effect of amphotericin B clearly depends on the amount of cholesterol that is introduced (Figs. 5—7). These results can be explained by an adhesion mechanism only if there is an exchange of cholesterol between adhering vesicles and the filter. This possibility cannot be excluded, although we obtained evidence for the absence of a rapid exchange between suspended vesicles with and without cholesterol, even when these were aggregated in the presence of high concentrations of Ca^{2+} (see Results). Moreover, the experiment of Fig. 7 is difficult to explain in this context.

Thirdly, it was found that the dissipation of the photopotential by gramicidin varied significantly depending on whether the same vesicles were associated with negatively charged filters in the presence of Ca^{2+} or were associated directly with positively charged filters (Table III). This result would be unlikely if the vesicles were to adhere to the filter.

Finally, the sign of the photopotential generated by bacteriorhodopsin is independent of the net direction of H^+ movement in bacteriorhodopsin vesicles in suspension (Table IV). This result can be explained by an adhesion mechanism, if the vesicle preparation consists of a mixture of two types of vesicles, pumping protons in opposite directions and if there were a selective adhesion of vesicles that accumulate protons upon illumination. It has been found that bacteriorhodopsin vesicles, prepared according to the procedure of Happe et al. [42], indeed consist of a mixture pumping protons in different directions [43]. Using a similar vesicle preparation as used in the experiment of Table IV, prepared at pH 2.5, it was found, however, that dimethyloxazoledine-2,4-dion was accumulated by a factor 15—20-fold greater than methylamine [43]. These two compounds were used as probe molecules for vesicles that extrude protons and accumulate protons, respectively, upon illumination. Based on this difference, and on the fact that the net charge of the vesicles is largely determined by the negatively charged cardiolipin, we believe that a preferential adhesion of proton-accumulating vesicles is very unlikely.

All experiments reported here can be explained by assuming that bacteriorhodopsin vesicles fuse with the hexadecane phase of the filter. A continuity between these two structures is indicated by the experiments in which the effects of polyene antibiotics were studied after selectively introducing cholesterol (Figs. 5 and 6). After associating cholesterol-containing vesicles, the effect of amphotericin B on the photopotential was found to decrease when the incubation time between removal of unassociated vesicles and the addition of anti-

biotic was increased (data not shown). This implies that cholesterol is introduced initially into the surface layer of the hexadecane phase, and subsequently diffuses to deeper parts of this phase. In the experiment with phospholipase A_2 (Fig. 3) the decrease in photopotential is probably due to the layer between compartments I and III containing elevated levels of lysophospholipids and free fatty acids, which are known to increase the passive permeability of membranes [45,46]. A complete breakdown of this layer may be prevented by an exchange diffusion of the hydrolysis products from this layer with phospholipids from the bulk hexadecane phase, which can function as an enormous reservoir. If we assume continuity between the vesicle bilayer and the hexadecane phase we can also explain the different effect of gramicidin on the photopotential after association of vesicles to negatively charged and positively charged filters, respectively (Table III). In these cases bacteriorhodopsin will pump protons across a layer having different net charge. Since gramicidin forms cation-selective channels [44], its effect on the photopotential will be different in both systems, simply because of electrostatic attraction or repulsion of the cations that have to cross the membrane via the pore. A similar explanation has been given for the effect of membrane-surface charge on the valinomycin-induced potassium permeability in cells and liposomes [47].

The experiment with bacteriorhodopsin vesicles, which have an opposite net direction of light-dependent H^+ movement (Table IV), may be explained by a fusion mechanism if it is assumed that during fusion there is a reorientation of bacteriorhodopsin to a preferred position at the filter-water interface; photopotentials always have the same sign.

In addition, it has been found that hydrocarbons, e.g. decane and hexane, promote mutual fusion of lecithin liposomes [9]. On the basis of the above evaluation we conclude that most probably a fusion of bacteriorhodopsin vesicles with the filter takes place on association. It is more difficult, however, to conclude whether the compartment enclosed by the vesicle bilayer is

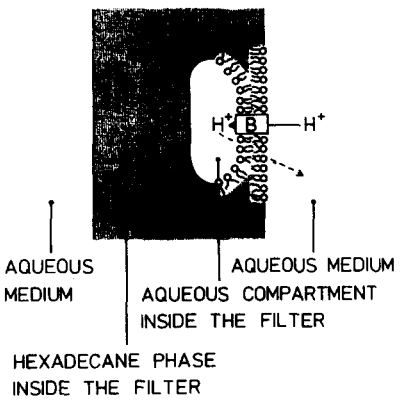


Fig. 8. Model for the association of bacteriorhodopsin with a lipid-impregnated filter. The aqueous compartment inside the filter is referred to as compartment III in the text. The aqueous media on the right-hand and the left-hand side of the filter are compartments I and II, respectively. B, bacteriorhodopsin.

retained during fusion or whether this compartment is lost. Since aqueous compartments are already present in the hexadecane phase of the filter, the effect of gramicidin on the photopotential does not constitute proof for the conclusion that the vesicular structure is retained [13,17]. It is possible that these same compartments constitute compartment III, which is the acceptor reservoir into which bacteriorhodopsin pumps protons upon illumination. This has been illustrated schematically in Fig. 8. Apart from the pathway of protons, indicated in the figure, high-resistance connections between the three aqueous compartments exist across the hydrophobic phase. Although, from a molecular point of view, this model differs from the ones presented by other groups [13, 17,18], its electrical implications are largely equivalent.

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