RECONSTITUTION OF BACTERIORHODOPSIN IN A MILLIPORE FILTER SYSTEM

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Received 14 February 1977

1. Introduction

At present it is generally accepted that bacteriorhodopsin, which is the only protein present in purple membranes of Halobacteria [1], functions as a light-driven proton-pump [2]. The conversion of light-energy into chemiosmotic energy by this protein has been also demonstrated in model membrane systems, e.g., in liposome systems where bacteriorhodopsin can be incorporated either by sonication procedures [3,4] or by methods which employ detergents [5,6]. Incorporation of bacteriorhodopsin into liposomes together with a second protein, e.g., mitochondrial or bacterial adenosine triphosphatase complex [5,7] or cytochrome *c* oxidase [8], yielded vesicles in which the reaction, catalysed by the second protein, could be influenced by illuminating the vesicle suspension.

Since dispersions of purple membranes are rather stable in decane solution, bacteriorhodopsin was easily reconstituted in black-film systems [9–12]. An improved method has been put forward by the group of Skulachev, who developed the so-called planar membrane system. Bacteriorhodopsin-containing vesicles can be associated with this thick membrane in the presence of Ca^{2+}-ions [11–13].

In the present paper experiments are described using a related system, in which the planar membrane has been replaced by lipid-impregnated Millipore filters. The two big advantages of this system are its high stability and the possibility to increase drastically the membrane area.

Abbreviations: FCCP carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone, BRh bacteriorhodopsin.

2. Materials and methods

Purple membranes [14] and soya-bean phospholipids [15] were isolated according to procedures already described.

Incorporation of bacteriorhodopsin into lipid vesicles was done as follows: soya-bean phospholipids, dissolved in chloroform, were evaporated to dryness in a round-bottom flask. After addition of purple membranes, suspended in 150 mM KCl, and dilution to the desired protein concentration with 150 mM KCl, the lipids were dispersed by mixing on a Vortex mixer in the presence of small glass-beads. This suspension subsequently was sonicated in a MSE ultrasonifier for 30- or 60-times 15 s, with 45 s intervals without sonication, at 20 kHz, 4 μm amplitude, 0°C, and under argon atmosphere. The final lipid concentration was 10 mg/ml.

Millipore filters (pore size 0.15 μm, 0.15 mm thick) were impregnated with soya-bean phospholipids by dipping the filter in a hexadecane solution of the lipids (150 mg/ml). After removal of excess lipid solution the filter was clamped between two 20 ml Teflon-vessels with an aperture of 12 mm diameter. Electrical measurements were carried out with a circuit as described by Mueller et al. [16] using two calomel electrodes (Radiometer, Copenhagen, Type K 4112), connected with a Keithley 610 C electrometer and a recorder. Illumination from one side was achieved with a 250 W projector lamp and fiber optics.

Valinomycin and nigericin were gifts from Eli Lilly Co. FCCP was a gift from Dr P. G. Heytler.

3. Results and discussion

Filters, prepared as described in Materials and methods, and bathed on both sides with 20 ml 50 mM $CaCl_2$, 75 mM KCl solution, usually have a resistance of about 4–6 X 10^8 Ω. Illumination of the filters in the absence of added bacteriorhodopsin-containing lipid-vesicles (BRh-vesicles) did not give a photopotential. Because filters soaked in hexadecane solutions are fairly transparent it is possible to generate photopotentials by illuminating the bacteriorhodopsin through the filter.

Addition of BRh-vesicles to one of the compartments gives rise to the generation of a photopotential, upon illuminating the filter, which disappears upon turning off the light. Assuming that the generation of the potential is a good measure for the 'association' of the vesicles with the lipid-impregnated filter, fig.1 shows that the association is a time-dependent process. Initially the rate of association is very fast but tends to reach a maximum and levels off. Most likely this is due to a maximal loading of the filter surface with BRh-vesicles. How the vesicles are associated with the filter is unknown. However, when BRh-vesicles are also added to the second compartment the photopotential, generated during illumination of the filter, rapidly decreases (fig.1). The rapid decrease of the potential indicates that the binding of BRh-vesicles to this second surface of the filter is not significantly influenced by pre-incubation of the filter with vesicles present on the other side of the filter. There were no significant differences between experiments where the vesicles were first added to the front compartment and secondly to the rear compartment, and experiments where the sequence of additions was reversed.

That the vesicles have to be associated with the filter in order to generate photopotentials, is suggested by the requirement for Ca^{2+}-ions in the incubation medium. A similar observation has been reported for experiments with planar membrane systems [12,13]. This can also be demonstrated more directly by removing the unassociated BRh-vesicles after a given

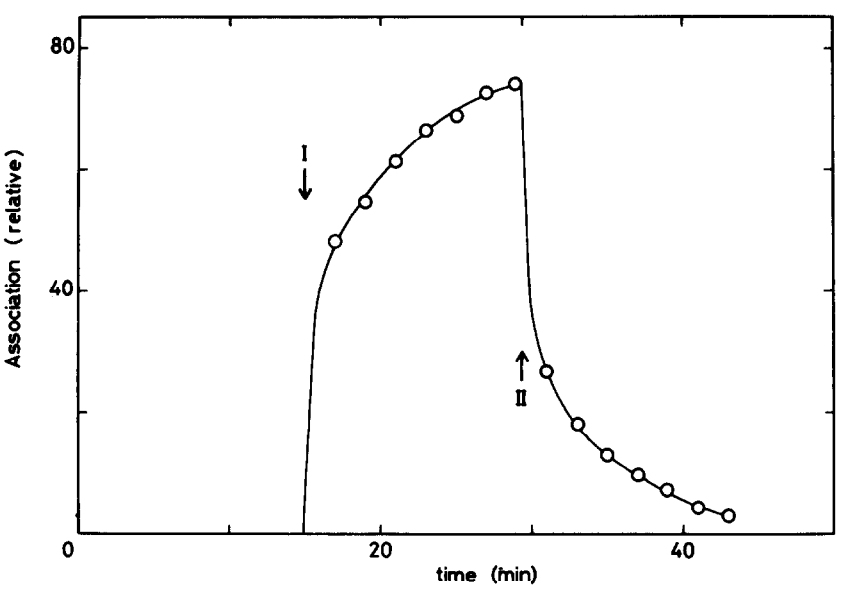

Fig.1. Time-dependence of the association of bacteriorhodopsin-containing vesicles with lipid-impregnated Millipore filters and asymmetry of the photo-effect. A lipid-impregnated filter was prepared as described in Materials and methods. Both Teflon-compartments were filled with a 50 mM $CaCl_2$, 75 mM KCl solution. Bacteriorhodopsin-containing vesicles were added to the rear and front compartment at the moments indicated by the first and second arrow, respectively. The protein/lipid ratio of the vesicles was 1/4 (w/w). Vesicles were added to a final concentration of 0.125 mg soya-bean phospholipids/ml. The association of the BRh-vesicles with the filter is expressed as the potential evoked by illuminating the filter for 5 s at the moments indicated by circles.

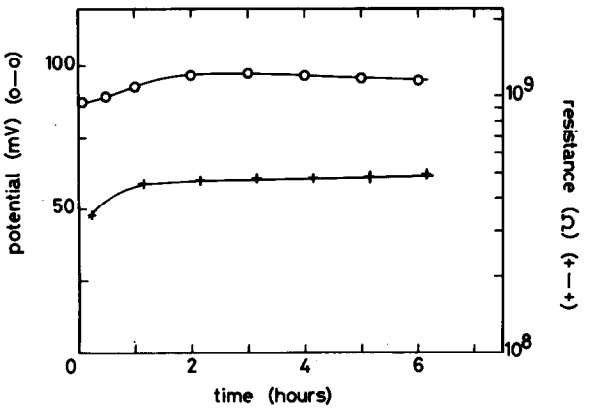

Fig.2. Stability of the association of bacteriorhodopsin-containing vesicles with lipid-impregnated Millipore filters. A lipid-impregnated filter, prepared as described in Materials and methods, was incubated for 15 min on one side with BRh-vesicles (protein/lipid ratio = 1/8 (w/w); final concentration of the vesicles in a medium of 30 mM $CaCl_2$, 105 mM KCl was 0.07 mg protein/ml). After removal of unassociated vesicles both compartments were filled with 50 mM $CaCl_2$, 75 mM KCl. The generation of a photopotential was measured as a function of time by illuminating the filter for 15 s at the moments indicated by circles. Resistance of the filter was measured as described in Materials and methods.

incubation period. Figure 2 shows an experiment in which the vesicle suspension was replaced by $CaCl_2$–KCl solution. It can be seen that the photopotential, generated by illuminating the filter, shows a slight increase during the first hour after removal of the BRh-vesicles. During this period we also measured the most drastic change in the resistance of the filter (fig.2). The photopotential, generated during illumination of the filter for 15 s, reached a maximum about 2 h after removal of the vesicles and remained nearly constant for the next 2 h. About 4 h after removal of the vesicles, the potential started to decrease slightly with time. This may be due to denaturation of the protein or to a slow release of the vesicles from the filter. From this experiment it can be concluded that the BRh-vesicles are tightly associated with the filter. Moreover, fig.2 shows that after association of BRh-vesicles with the Millipore filter very stable lipid-barriers with high electrical resistances are obtained.

Concerning the way BRh-vesicles are associated with the lipid-impregnated Millipore filters or with planar membranes, there is probably no fundamental difference between the two systems. By scanning planar membranes with a thin light-beam Drachev et al. found that especially the thick membrane borders contribute to the photo-effect [12]. The similarity between the two systems is supported also by the observation that shunting of the system with an external resistance or the addition of a protonophore like FCCP produces qualitatively similar effects. Figure 3A shows that both the amplitude and the form of the photo-effect are affected by shunting the membrane. The addition of FCCP, in a concentration range that reduces the resistance of the lipid-impregnated filter, only decreases the amplitude of the photo-effect but does not change its form (fig.3A). In this respect it is of interest to note that in the case of black lipid membranes, where bacteriorhodopsin was incorporated by dispersing purple membranes in the lipid-containing decane solution, the addition of carbonyl cyanide *m*-chlorophenylhydrazone did change the form of the photo-effect [9]. This difference may be related to differences in the methods of reconstitution of bacteriorhodopsin.

Besides the effect of uncouplers we tested how nigericin and valinomycin influenced the photo-effect (fig.3B and C). Nigericin causes a slight but significant increase of the photo-effect. This indicates that in some part of the circuit pH-gradients are generated during illumination of bacteriorhodopsin. Assuming a constant $\Delta\bar{\mu}H^+$, the nigericin-mediated K^+–H^+ exchange results in a decrease of the ΔpH and in an increase in $\Delta\psi$. In the model proposed by Drachev et al. [12] this observation can be explained easily. In this model the BRh-vesicles are associated with the planar membrane in such a way that the vesicles retain their enclosed aqueous compartment. Thus, light-induced pH-changes across the vesicular membrane may occur. Figure 3B also shows that the presence of both valinomycin and nigericin decreases the amplitude of the photo-effect and lowers the resistance of the filter. Thus, qualitatively similar results are obtained as in the case where FCCP was added (fig.3A). When only valinomycin was added a change in the form of the photo-effect was observed together with a decrease in the amplitude (fig.3C), similar to the situation of shunting the membrane (fig.3A). To explain these effects, experiments are in progress to get more insight in the way the BRh-vesicles are associated with the filter.

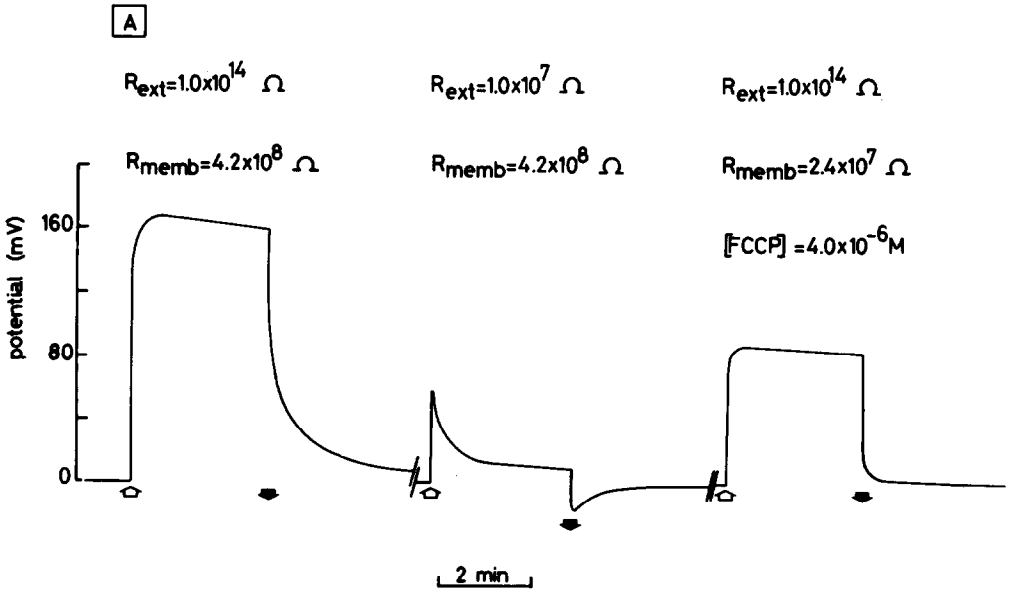
A
$R_{ext}=1.0x10^{14}$ Ω
$R_{memb}=4.2x10^{8}$ Ω
$R_{ext}=1.0x10^{7}$ Ω
$R_{memb}=4.2x10^{8}$ Ω
$R_{ext}=1.0x10^{14}$ Ω
$R_{memb}=2.4x10^{7}$ Ω
[FCCP] $=4.0x10^{-6}$M
potential (mV)
160
80
0
2 min

Fig.3A

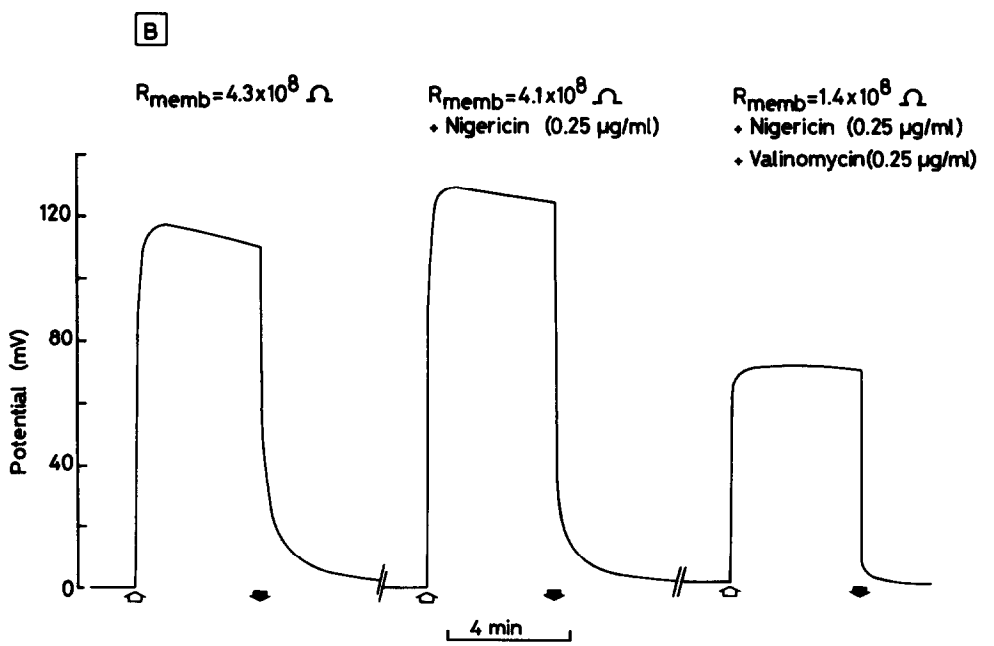
B
$R_{memb}=4.3x10^{8}$ Ω
$R_{memb}=4.1x10^{8}$ Ω
+ Nigericin (0.25 µg/ml)
$R_{memb}=1.4x10^{8}$ Ω
+ Nigericin (0.25 µg/ml)
+ Valinomycin(0.25 µg/ml)
Potential (mV)
120
80
40
0
4 min

Fig.3B

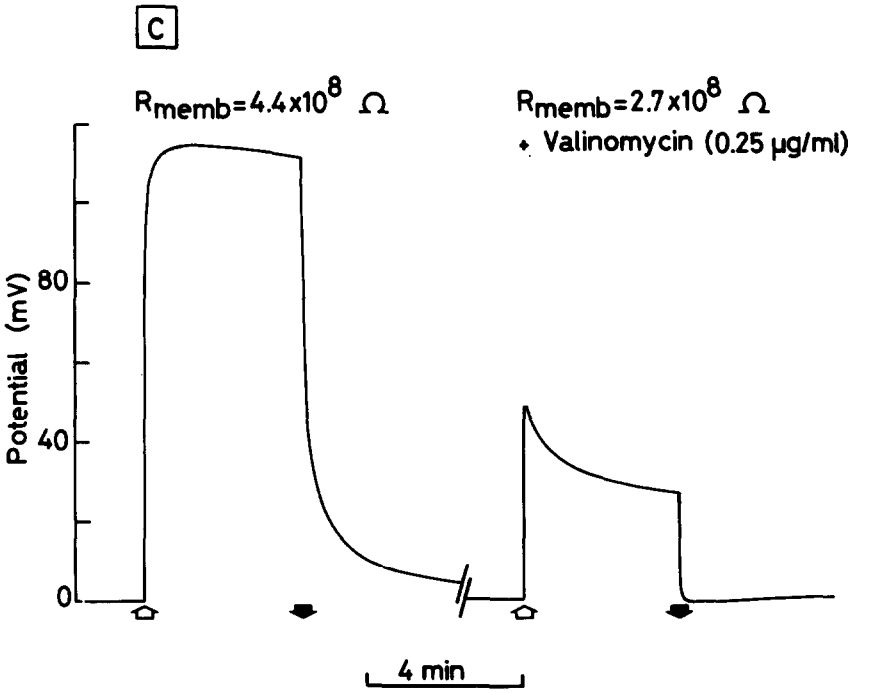

Fig.3C

Fig.3. The effect of ionophores on the generation of a photopotential. Filters were prepared as described in the legend of fig.2. After removal of the vesicles both compartments were equilibrated with 50 mM $CaCl_2$, 75 mM KCl for 1 h before starting the experiments. After addition of an ionophore to the compartment where the BRh-vesicles had been present, the system was equilibrated for at least 15 min before measuring the effect of the ionophore on the photopotential. Illumination of the filter was started and finished at the moment indicated by (⇧) and (⇩), respectively. (R_{memb}) Resistance of the filter. (A) Effect of an external shunting resistance (R_{ext}) and of an uncoupler. (B) Effect of nigericin in the absence and presence of valinomycin. (C) Effect of valinomycin.

Summarizing, it can be concluded from the above experiments that the association of BRh-vesicles with lipid-impregnated Millipore filters probably is similar to the association with planar membranes [12,13]. The Millipore filter system, however, has some attractive advantages, e.g. a higher stability and a large increase in the 'membrane' area. Moreover, higher photo-effects have been observed than those reported for the planar membrane system. Values of up to 215 mV have been obtained with the present method.

Acknowledgements

The present investigation was carried out with financial aid from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands Foundation for Chemical Research (S.O.N.).

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