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NYSTATIN-INDUCED INCREASE IN PHOTOCURRENT IN THE SYSTEM 'BACTERIORHODOPSIN PROTEOLIPOSOME/BILAYER PLANAR MEMBRANE'

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Proteoliposomes were reconstituted from bacteriorhodopsin sheets, asolectin and cholesterol with or without nystatin. Bacteriorhodopsin-mediated electrogenesis was monitored using (1) a proteoliposome suspension and phenyldicarbaundecaborane (PCB^-) probe or (2) proteoliposomes associated with planar bilayer membrane and orthodox electrometer techniques. In the light, PCB^- was shown to be taken up by proteoliposomes. The PCB^- uptake was inhibited by addition of nystatin to an incubation mixture with proteoliposomes if they were reconstituted in the presence of nystatin. Extraproteoliposomal nystatin was without influence if nystatin was omitted from the reconstitution mixture. The nystatin-containing proteoliposomes were associated with a planar bilayer asolectin membrane in the presence of Ca^{2+}. It was found that in such a system, bacteriorhodopsin generated a photocurrent charging the proteoliposome-containing (*cis*-side) compartment negatively and the *trans*-side compartment positively. The photoresponse was shown to be increased several-fold by addition of nystatin to the *trans*-side solution. Nystatin addition was ineffective if proteoliposomes were reconstituted without nystatin. Taking into account that nystatin forms ion-permeable pores in a membrane only if present on both sides of the membrane and that this membrane is bilayer, one can explain the above data assuming that (1) the intraproteoliposomal solution does not mix with the extraproteoliposomal one when proteoliposomes are attached to a planar black membrane and (2) the attached proteoliposomes are separated from the *trans*-side bathing solution by a bimolecular membrane. If this is the case, nystatin in the *trans*-side bathing solution and inside the attached proteoliposome can form pores across that part of the planar membrane which separates the proteoliposome interior from the *trans*-side solution. Through these pores, H^+ (pumped by bacteriorhodopsin from the *cis*-side solution into the proteoliposome interior) or some other intraproteoliposomal ions can be equilibrated with those in the *trans*-side solution. As a result, the bacteriorhodopsin-generated photocurrent increases.

Introduction

Generation of a photoelectric potential difference and photocurrent by bacteriorhodopsin was first directly measured in 1974 [1]. A planar phospholipid membrane was formed from a mixture of a decane solution of phospholipids and bacteriorhodopsin-containing fragments of *Halobacterium halobium* membrane (bacteriorhodopsin sheets). Illumination was found to induce a voltage and current across the planar membrane which were measured with two electrodes separated by this membrane. Later, the bacteriorhodopsin-mediated photoelectric effect was reproduced in many laboratories [2–8]. Now it is generally accepted that the effect is due to the operation of bacteriorhodopsin as an electrogenic light-driven H^+ pump (for a review, see Ref. 9).

Abbreviation: PCB^-, phenyldicarbaundecaborane anion

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Analysis of the mechanism of the bacteriorhodopsin photoeffect revealed that the thickness of the planar membrane is not critical, and the response can be demonstrated in black (bimolecular) membranes [2,10,11] as well as in a system composed of a several-millimetre thick layer of a decane solution of phospholipids, covering an electrolyte solution, with electrodes immersed into the bulk lipid and water phases [12]. In the latter 'biphasic' system, it was shown that the photopotential is completely inhibited by K^+ if a low amount of gramicidin A is present [12]. Since gramicidin A is known to form K^+-permeable transmembrane channels only when the membrane is bimolecular, the conclusion was made that the photoeffect in the biphasic system is caused by H^+ translocation from the bulk water phase into a water cavity between the bulk lipid phase and a bacteriorhodopsin sheet situated in the interface. Similar relationships were described when thick phospholipid membranes or phospholipid-impregnated filters and films were used as a support for bacteriorhodopsin sheets or proteoliposomes [13]. This means that in all these systems, the photocurrent was limited by a high resistance of the passive support to which active bacteriorhodopsin-containing membranes were attached.

Bamberg and co-workers [8] observed an increase in photocurrent on addition of gramicidin A or a protonophorous uncoupler to the bathing solution on the bacteriorhodopsin-free side of a bilayer planar membrane treated with bacteriorhodopsin sheets. This effect can be explained by a decrease in the resistance of the supporting planar membrane. Unfortunately, gramicidin as well as protonophores can also decrease the resistance of those parts of the planar membrane that are not covered with the sheets. Besides, sheet membrane shunting may take place if gramicidin reaches this membrane. Both these effects must decrease the photocurrent.

In this paper, we tried to overcome the above limitations, using nystatin to decrease specifically the resistance of the supporting planar membrane only in those regions that are covered with bacteriorhodopsin proteoliposomes.

Nystatin is an agent forming channels across cholesterol-containing membranes when present on both sides of the membrane [14]. It cannot decrease the resistance of cholesterol-free membranes.

Experiments are described below showing that bacteriorhodopsin photocurrent can be greatly increased if a nystatin solution and nystatin-containing proteoliposomes are added on opposite sides of the black phospholipid membrane.

Materials and Methods

Black planar membranes were formed on a 1 mm^2 aperture in the septum dividing a Teflon chamber into two equal compartments. The membrane-forming decane solution of asolectin contained 50 mg phospholipid/ml. Bacteriorhodopsin proteoliposomes were reconstituted by sonication of a mixture of bacteriorhodopsin sheets prepared from *H. halobium*, strain R_1 (see Ref. 15) (1 mg protein/ml, 45 mg asolectin/ml, 5 mg cholesterol/ml and 2% sodium cholate and, when indicated, $1 \cdot 10^{-4}$ M nystatin). Sonication was carried out three times during 1 min at 1°C.

Before addition to the mixture for proteoliposome reconstitution, cholesterol was sonicated during 5 min in 0.05 M Tris-HCl, pH 7.1.

After sonication, the mixture was dialyzed twice for 8 and 18 h against 0.05 M Tris-HCl, pH 7.1, with or without $1 \cdot 10^{-4}$ M nystatin. The resulting proteoliposomes were stored at 1°C.

PCB^- uptake was measured in the proteoliposome suspension using the planar phospholipid membrane as a PCB^--sensitive electrode [16].

Proteoliposome incorporation into the planar membrane was carried out in the presence of 0.03 M $CaCl_2$ [11]. Electric responses to continuous illumination and flashing light were measured as described in Refs. 11 and 17, and Refs. 18 and 19, respectively. As light sources, we used a 20 W tungsten lamp or a neodymium Q-switched laser of double light frequency ($\lambda = 530$ nm, $t_{1/2} = 15$ ns, light impulse energy 5 mJ). For measurement of the fast kinetics of the electrogenic response, see Ref. 13.

Results and Discussion

In Fig. 1, PCB^- responses of two kinds of bacteriorhodopsin proteoliposomes are shown. It is seen that addition of nystatin to the sample with

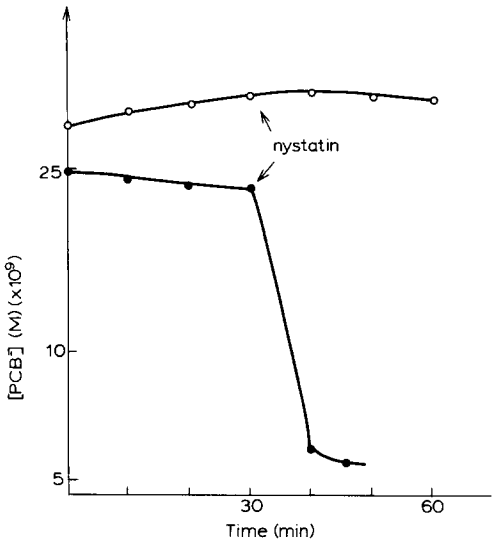

Fig. 1. Effect of nystatin addition on the PCB^- photoresponse in a suspension of bacteriorhodopsin proteoliposomes with or without nystatin inside. Incubation mixture contained 50 mM Tris-HCl, pH 7.1, $1 \cdot 10^{-6}$ M PCB^- and proteoliposomes (0.01 mg/ml). Addition, $1 \cdot 10^{-5}$ M nystatin. Abscissa, incubation time. Ordinate, PCB^- concentration in the incubation mixture. (○) Control, (●) nystatin inside proteoliposomes.

nystatin-containing proteoliposomes results in strong inhibition of the light-induced PCB^- uptake. This observation indicates that nystatin proteoliposomes contain enough nystatin inside to shunt the bacteriorhodopsin photopotential if nystatin is also present outside. In control (nystatin-free) proteoliposomes, no inhibition by external nystatin was revealed during at least 30 min. This means that there is no fast equilibration of nystatin across the proteoliposomal membrane.

Figs. 2–5 show data of the experiments in the system 'proteoliposome/bilayer planar membrane'. The process of proteoliposome association with the planar membrane was followed by measuring electric responses to 15-ns laser flashes and to continuous illumination. As can be seen in Fig. 2A and B, a flash inducing a single turnover of bacteriorhodopsin results in an electric potential difference composed of micro- and millisecond phases. During 3 h incubation, the following characteristic changes in the flash-induced response were revealed: (i) a great increase in the microsecond phase amplitude, and (ii) some increase in the millisecond phase amplitude, which proved to be not so large as that in the microsecond phase. The latter effect was found to be due to acceleration of the photopotential decay (cf. Fig. 2A and B). The rise of the photopotential amplitude is explained by proteoliposome association with the planar membrane. This process was shown to be completed in several hours [11]. As to the decay time shortening, it is most probably due to some increase in the conductance of the planar membrane (see below, Fig. 5). Such an effect was always present when nystatin was added on the *trans*-side of the planar membrane treated on the *cis*-side with nystatin proteoliposomes. Apparently, contaminations by extraproteoliposomal nystatin in the proteoliposome suspension proved to be sufficient to induce a rise of the conductance of proteoliposome-free regions of planar membrane. Acceleration of the photopotential decay hinders accurate measurement of the size of the millisecond phase, since the rise time of this phase becomes of the same order of magnitude as the decay time. As to the microsecond phase amplitude, it can still be accurately estimated, so that only this parameter was taken into account to follow proteoliposome incorporation.

To monitor a nystatin-induced increase in photocurrent, the response to continuous illumination was used, since it is continuous activity of bacteriorhodopsin, rather than its flash-actuated single turnover, that can be limited by the resistance of the supporting membrane. The continuous light-induced photoeffects are shown in Fig. 2C and D.

In Fig. 3 the time courses of the flash- and continuous light-induced electric responses are compared. In both cases, the process of the photocurrent increase was found to require more than 3 h. The amplitude of the flash effect which, as we assume, is a simple function of the saturation degree of the planar membrane surface with proteoliposomes, increases monotonically throughout the duration of the experiment. As to continuous light, its photoeffect seems to have a more complicated (sigmoid) time course. Apparently, proteoliposome incorporation is followed by some other process, which is required to increase the steady-state level of photocurrent. This may be formation of nystatin half-channels by intraproteoliposomal nystatin molecules, or lateral diffu-

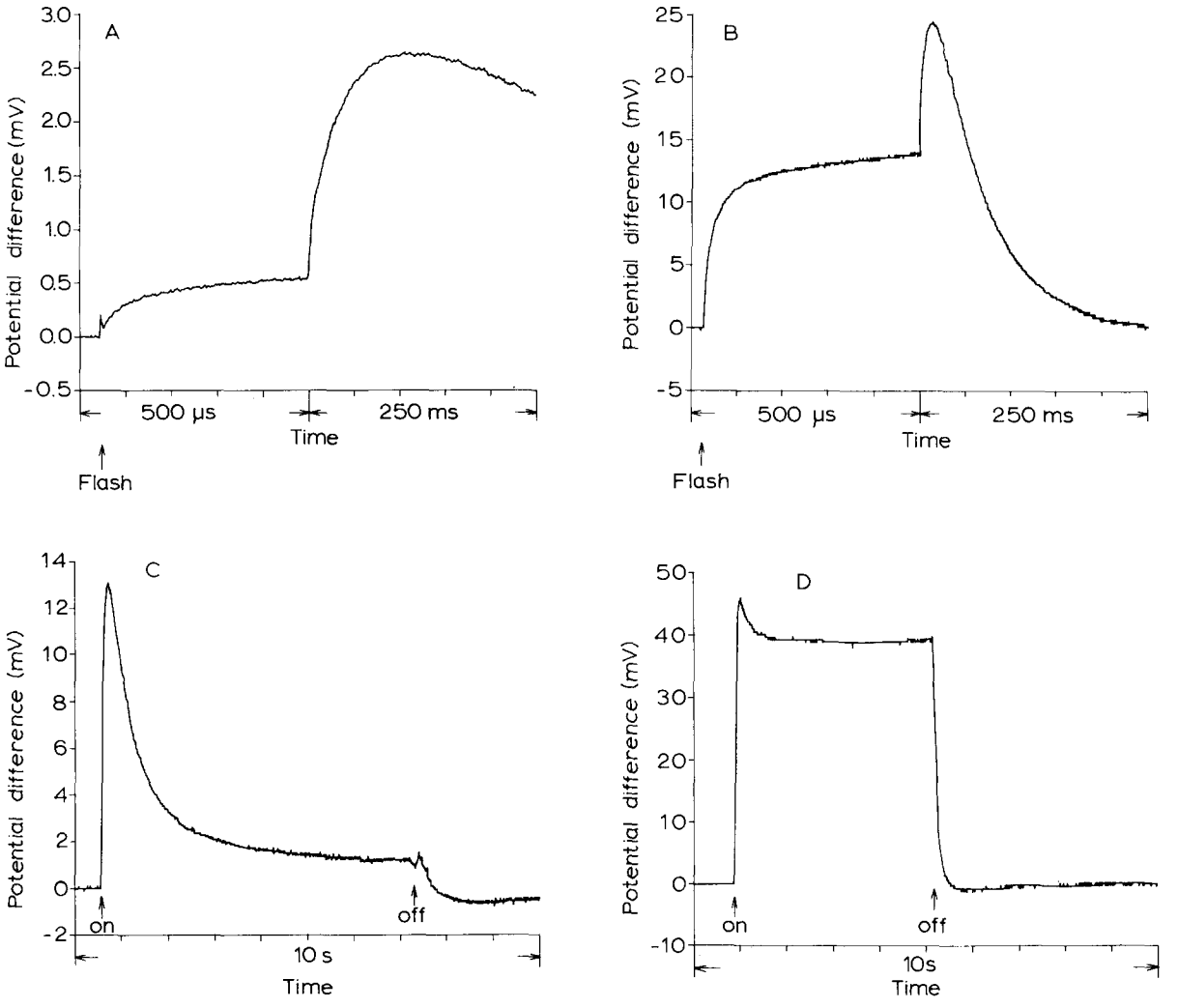

Fig. 2. Photoelectric responses induced by a 15 ns laser flash or continuous illumination in a proteoliposome/bilayer planar membrane system. Nystatin was present both inside the proteoliposomes ($1 \cdot 10^{-4}$ M) and in the *trans*-side bathing solution ($1 \cdot 10^{-5}$ M). Bathing solutions contained 50 mM Tris-HCl, pH 7.1, and 30 mM $CaCl_2$ on both sides of the planar membrane and nystatin proteoliposomes on the *cis* side. (A, B) 15-ns laser flash, (C, D) continuous illumination. The planar membrane was treated with proteoliposomes for 50 (A), 190 (B), 70 (C) and 145 min (D).

sion of cholesterol from the proteoliposomal membrane to the planar one. (For sensitization of a cholesterol-deficient planar membrane to nystatin by means of cholesterol-containing liposomes see Refs. 21 and 22).

The time courses of electric responses to continuous illumination in nystatin-containing and nystatin-free proteoliposomes are given in Fig. 4. In the latter case, we supplemented the *cis*-side solution with nystatin at a concentration of $1 \cdot 10^{-6}$ M that had been added as a contamination to nystatin proteoliposomes. So the samples differed only in the presence or absence of nystatin inside proteoliposomes. Nystatin addition on the *trans*-side was made at zero time. It is shown in the figure that the photocurrents are much higher in the case of the nystatin proteoliposomes than in the control preparation. This could be predicted if we assumed that proteoliposomes were associated with the planar membrane surface without release

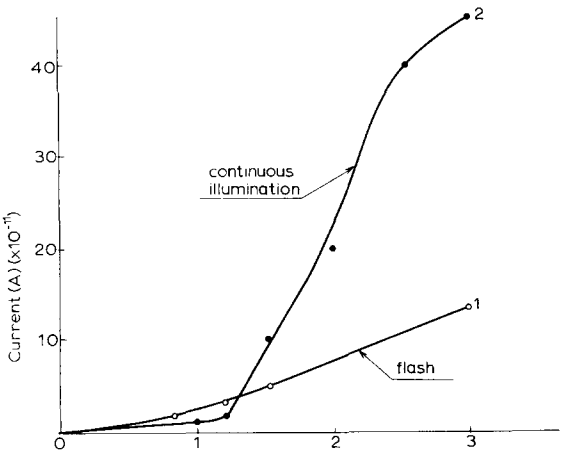

Fig. 3. Comparison of time courses of photoelectric responses to flash and to continuous illumination in a proteoliposome/bilayer planar membrane system. For flash responses, current was measured 0.5 ms after the flash. For continuous light responses, plateau values of the current were used. For conditions, see Fig. 2.

of the intraproteoliposomal solution into the bulk incubation medium. If this is the case, intraproteoliposomal nystatin and nystatin in the *trans*-side bulk solution can form ion-permeable channels across the planar membrane in the place of the proteoliposome attachment. This must entail a specific decrease in the electric resistance of this part of the planar membrane and, as a consequence, a continuous photocurrent increase.

In the nystatin-free proteoliposomes, no rise of the photoresponse is observed during the first 20 min of incubation. Then it slowly increases, apparently due to diffusion of nystatin from the *trans*-side solution into the intraproteoliposomal space.

In the experiment shown in Fig. 5, nystatin was added to the *trans*-side solution 42 min after proteoliposome addition to the *cis*-side solution. During this period, some proteoliposomes were already associated with the planar membrane so that nystatin half-channels can be formed in the planar membrane regions covered by nystatin-containing proteoliposomes. Under these conditions, the *trans*-side nystatin induced a photocurrent increase without any lag phase. In the sample with the nystatin-free proteoliposomes, nystatin addition was without any effect in the given time scale.

The above data can be explained in terms of the scheme shown in Fig. 6. According to this scheme, proteoliposomes are associated with the supporting planar membrane in such a fashion that they retain their intraproteoliposomal solution. So, the joint action of nystatin localized inside proteoliposomes and nystatin added to the *trans*-side bulk solution results in the formation of channels connecting the intraproteoliposomal and *trans*-side water. These channels allow the H^+ that is pumped from the *cis*-side solution into proteoliposomes to

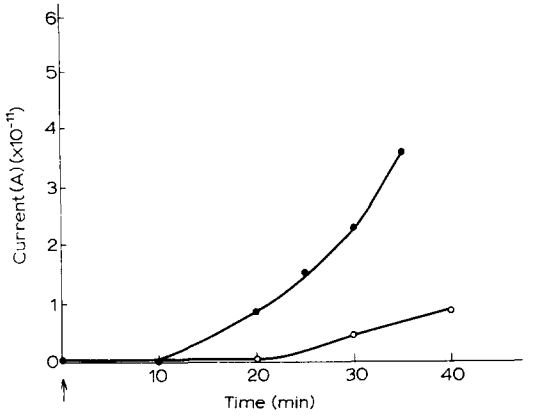

Fig. 4. Requirement of intraproteoliposomal nystatin for a photocurrent increase in a proteoliposome/bilayer planar membrane system with nystatin on the *trans* side. Continuous illumination. For conditions, see Fig. 2. (○) Control, (●) nystatin.

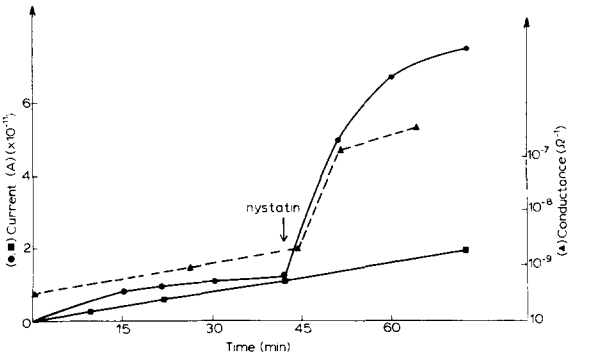

Fig. 5. Photocurrent and conductance changes induced by nystatin addition on the *trans* side of a proteoliposome/bilayer planar membrane system. Conductance was measured in the dark in a sample with nystatin proteoliposomes. Continuous illumination. Addition, $1 \cdot 10^{-5}$ M nystatin on the *trans* side. For conditions, see Fig. 2. (■) Control, (●, ▲) nystatin inside proteoliposomes.

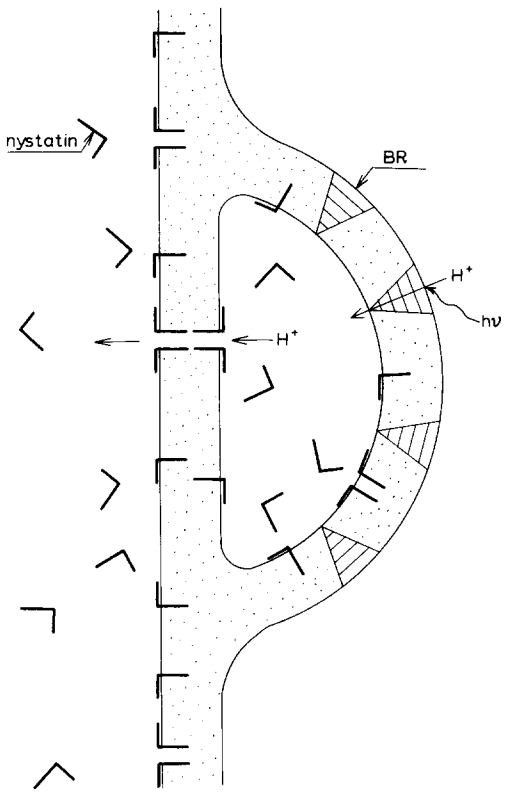

Fig. 6. A scheme explaining the nystatin-induced increase in bacteriorhodopsin-generated photocurrent. BR, bacteriorhodopsin.

be released into the *trans*-side solution. This causes an increase in the bacteriorhodopsin-generated photocurrent.

The above scheme differs from that proposed by Blok and Van Dam [6] who concluded that the intraproteoliposomal solution mixes with the bulk solution when proteoliposomes are associated with a supporting planar film (in their case, a phospholipid-impregnated cellulose nitrate filter instead of the black film used in our experiments, a fact that may account for the difference between their conclusions and ours). It is also different from the scheme proposed by Bamberg et al. [8] that assumes tight attachment of the bacteriorhodopsin membrane to the planar one with no water cavity between the two membranes. These versions fail to explain why *trans*-side nystatin increases the photocurrent only if there is nystatin inside proteoliposomes (see Figs. 4 and 5).

On the other hand, the scheme in Fig. 6 is in a good agreement with several pieces of evidence for the existence of an aqueous solution between the supporting and associated membranes, obtained in this group when systems other than black membranes were studied [11–13]. It should be noted in this context that an electron microscopic investigation of a photoelectrically active system, composed of a several-millimetre thick layer of a decane solution of phospholipids and an aqueous solution with bacterial chromatophores in the interface, revealed that chromatophores retain their closed vesicular structure when they are situated between bulk lipid and water phases [20].

In conclusion, it is shown that intraproteoliposomal nystatin is required to increase the bacteriorhodopsin-generated photocurrent in the system proteoliposome/planar black membrane with nystatin on the proteoliposome-free side of the planar membrane. This strongly supports the conclusion about the existence of intraproteoliposomal water in the studied system. Besides, the use of nystatin is promising for maximal increase in the bacteriorhodopsin photocurrent, since this can be done without shunting of the bacteriorhodopsin membrane and those parts of the planar membrane which are not covered with proteoliposomes.

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