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INTERACTION BETWEEN GRAMICIDIN-A AND BACTERIORHODOPSIN IN RECONSTITUTED PURPLE MEMBRANE

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Summary: The addition of gramicidin-A to reconstituted purple membrane, significantly inhibits light-induced proton movement. Kinetic analyses indicate that the treatment decreases the initial proton pumping rate (R_o), alters the interdependence (m) between the pumping process and its associated H^+ leak path (k_L-k_D), but has no detectable effect on the proton permeability associated with phospholipid bilayers in the dark (k_D). These results suggest that gramicidin-A, under the experimental conditions, interacts directly with bacteriorhodopsin in the membrane. This suggestion is supported by the findings that both the resonance Raman and circular dichroism spectra of bacteriorhodopsin are affected by the antibiotic.

Gramicidin-A was found to have the similar ionophoric activity as that of valinomycin in mitochondrial oxidative phosphorylation (1). However, unlike valinomycin which is a mobile ionophore, the concentration dependence of the transient conductance bursts caused by gramicidin-A in lipid membranes (2) suggests that it forms dimeric ion channel (3-5). Indeed, such an ordered structure has been observed by spectroscopic measurements (6-8). Although this channel formation may account for the ionophoric activity of gramicidin-A, the possibility of this antibiotic interacting directly with ion pumps in energy transducing membranes has not been investigated.

In this communication, the effects of gramicidin-A on light-induced proton movement of reconstituted purple membrane were studied and compared to results we obtained with valinomycin. The analyses on the kinetics of the proton movement in reconstituted systems and the spectroscopic measurements of native membrane indicate that gramicidin-A interacts directly with bacterior hodopsin.

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Abbreviation used: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

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Materials and Methods

Purple membrane was isolated from halobacterium halobium R_1 according to a published procedure (9). Bacteriorhodopsin was incorporated into sonicated soy bean phospholipid vesicles by the octylglucoside dilution technique of Racker et al. (10). A 4 ml solution (75 mM KCl, 1 mM Hepes, pH 8.0) containing 2 mg bacteriorhodopsin and 2.5% octylglucoside was mixed with an equal volume of sonicated suspension of phospholipids (120 mg) in the same solution. The mixture was incubated in the dark at 0°C overnight, and then diluted 20 times in volume with 150 mM KCl. The reconstituted purple membrane was collected by centrifugation at 100,000 x g for 45 min and was resuspended in 150 mM KCl to a proper concentration. Light-induced proton movement was measured by a pH meter at 25°C and analyzed as mentioned before (11). Excellent fits were obtained by assuming the growth phase of proton movement obeys the equation $\ln (1-\Delta/\Delta_s) = -k_L\ t\ [A]$. where Δ and Δ_s are the extent of proton uptake at time t after illumination and at the steady state, respectively, while k_L is the rate constant for proton leak under illumination. The release of protons in the dark stage follows the decay equation $\ln (\Delta/\Delta_s) = -k_D\ t\ [B]$, where k_D is a light-independent decay rate constant. The initial or the maximal proton pumping rate, R_o, is obtained from the steady-state approximation, $R_o = k_L\ \Delta_S\ [C]$. The circular dichroism and resonance Raman spectra were obtained with a JASCO J-41C Automatic Recording CD Spectrometer equipped with a data processor and a Spex 1401 double monochrometer with an Argon ion laser (Spectra Physics Model 165) operating at 514.5 nm-line, respectively.

Valinomycin and Hepes were obtained from Sigma Co.[1] Gramicidin-A and octylglucoside were purchased from Nutritional Biochemicals and Calbiochem, respectively. Soy bean phospholipids, asolectin, was from Associated Concentrates. All other reagents used were of analytical grade.

Results

Comparison of The Effects Between Gramicidin-A and Valinomycinon The Proton Movement: Haynes et al. (12) have shown that gramicidin, like valinomycin, induces an energy-linked cation for proton exchange in mitochondria. However, as shown in Figure 1, valinomycin and gramicidin have quite different effects on the reconstituted purple membrane vesicles. Valinomycin (Fig. 1B) nullifies the membrane potential (4) and hence increases the extent of proton translocatio On the other hand, gramicidin-A (Fig. 1C) decreases the steady state proton uptake, but it does not interfere with the ability of valinomycin to enhance the proton uptake (Fig. 1D).

Efficiency of Gramicidin-A in Inhibiting Proton Uptake: When gramicidin was added to the vesicles under steady-state illumination, the Δ_S decreased rapidly to a lower level. As shown in Figure 2, the observed proton translocation is diminished completely when about 75 μg of the antibiotic per mg

[1]Reference to brand or firm name does not constitute endorsement by U.S. Department of Agriculture over others of a similar nature not mentioned.

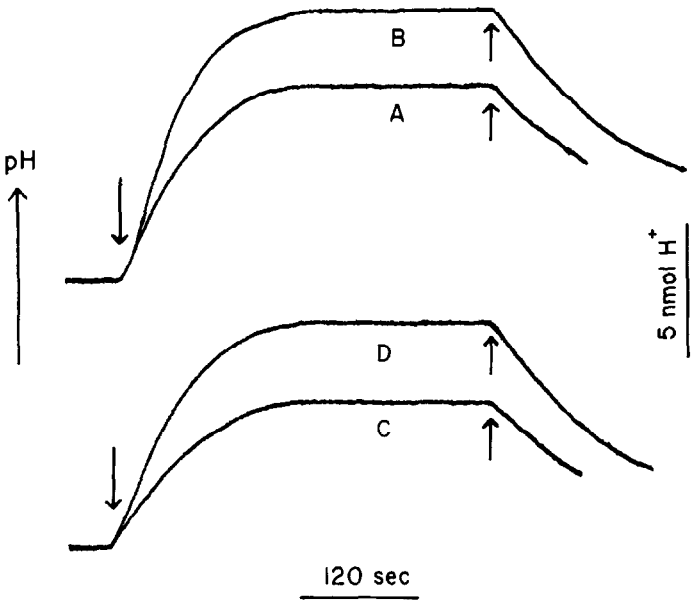

Figure 1. Effects of Valinomycin and Gramicidin-A on the Proton Movement. To a volume of 2.0 mL solution containing 150 mM KCl, 10 μg reconstituted bacteriorhodopsin, and the antibiotics with pH carefully adjusted to 5.3, red actinic light with an intensity of 190 Jm^{-2} sec^{-2} was used to initiate the proton uptake (↓). After the uptake reached steady state (Δ_S), illumination was then terminated (↑) and the protons were released back to the media. The extents of proton uptake for A (no antibiotics), B (400 ng valinomycin), C (30 ng gramicidin-A), and D (400 ng valinomycin + 30 ng gramicidin-A) are 598, 820, 530, and 731 n mol H^+/mg protein, respectively, at steady state.

of bacteriorhodopsin is added. The fact that this inhibition is independent of the composition of the medium and the light intensity, suggests that gramicidin-A interacts with the proton pump directly.

Kinetic Analysis on The Effects of Gramicidin-A: The observed depression of Δ_S indicates that either the maximal pumping rate (R_o), is decreased or the leak rate constants (k_L, k_D) are increased, or both, by the presence of the antibiotic. Thus, different amounts of gramicidin-A were added to reconstituted vesicles prior to illumination. The kinetics of the proton movement were then analyzed according to the scheme metioned. As shown in Figure 3, gramicidin-A has negligible effect on the rate constant k_D (-□-□-) which is known to be sensitive toward the changes in phospholipid environment (13). The rate constant (k_L-k_D) (-○-○-)associated with light-triggered proton decay decreases at lower concentration of gramicidin-A and then

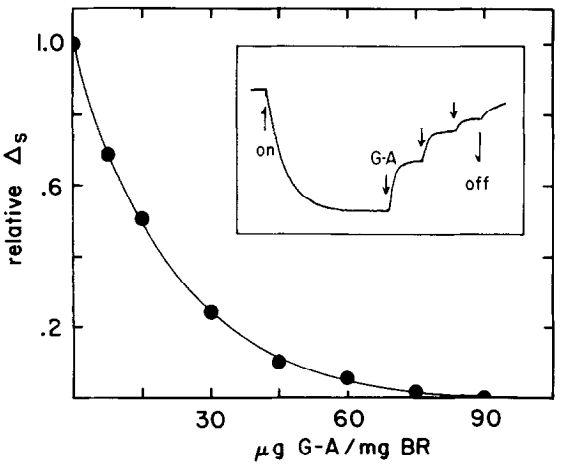

Figure 2. Efficiency of Gramicidin-A in Inhibiting the Proton Movement. Proton movement of reconstituted vesicles in the medium containing KCl and valinomycin was induced in the same manner as mentioned in Figure 1. After reaching steady-state, a volume of 1 μl of gramicidin in ethanol was added. As shown by the insert, this addition (↓) immediately depressed the steady state proton uptake to a lower level. Further additions of the antibiotic eventually abolished the uptake. The buffering capacity of the system was not affected by the additions. A similar inhibition efficiency of gramicidin-A was found in 150 mM KCl without valinomycin or in 150 mM NaCl. The concentration dependence of the inhibition remained unchanged by varing the light intensity from 135 to 265 Jm^{-2} sec^{-2}.

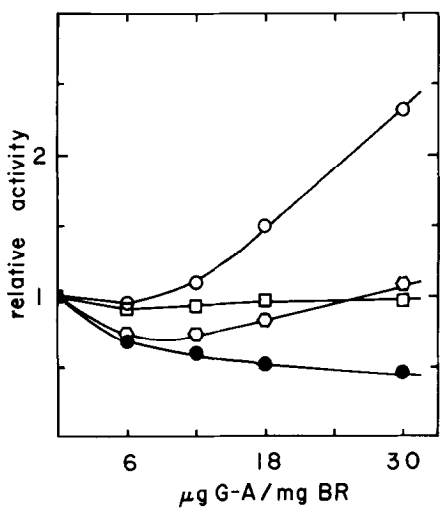

Figure 3. Effects of Gramicidin-A on the Kinetic Parameters. The kinetics of light induced proton movement of reconstituted vesicles pretreated with different amounts of the antibiotic in 150 mM NaCl were analyzed as described in Materials and Methods. The R_o, (k_L-k_D), k_D, and m of untreated system are 1870 n mol H^+/min. mg, 0.035 sec^{-1}, and 0.013 sec^{-1}, and 7.06 x 10^{-4} mg/n mol H^+, respectively, and are assigned the value of 1. ●, ○, □, and ○ represent the relative changes of R_o (k_L-k_D), k_D, and m, respectively; as a function of added gramicidin-A.

gradually returns to the same level as that of unmodified vesicles. The lack of enhancement on the leak rate constants suggests that there is no ion transfer channel formed by the antibiotic in the system with or without illumination. The initial proton pumping rate, R_o(-●-●-), which should be directly related to the primary photochemical cycle, decreases as the concentration of gramicidin-A increased. No detailed kinetic analyses were performed for vesicles treated with more than 30 μg/mg because Δ_S was too small for accurate evaluation of other parameters.

For a given reconstituted system, both R_o and (k_L-k_D) increase with increased light intensity (11). This relationship is expressed as $(k_L-k_D) = mR_o$ [D], where m is a constant independent of light intensity. This m, may be regarded as a regulatory constant indicating the link between R_o and (k_L-k_D) (14). The presence of gramicidin-A does not change the validity of equation [D] in the system (data not shown). However, the observed effects on R_o and (k_L-k_D) lead to the change of m (-O-O-) as depicted in Figure 3. It should be mentioned that the presence of valinomycin increases both the initial rate and the leak rate constants (data not shown).

Effects of Gramicidin-A on Spectroscopic Properties of Bacteriorhodopsin: The results mentioned in Figure 3 suggest strongly that gramicidin-A interacts directly with bacteriorhodopsin in the membrane. This suggestion was examined by spectroscopic methods. Because of the excessive light scattering of vesicles, the spectral measurements were instead made with isolated purple membrane in aqueous solution (without reconstitution). In the presence of the antibiotic, noticeable conformational changes of the retinal group in bacteriorhodopsin were observed. As shown in Figure 4, gramicidin causes a shift of the so called exciton peak (15) from 531 to 525 nm and an increase in the intensity of c-c stretching band at ~1170 cm^{-1} (16). These observations indicate a direct interaction between gramicidin and bacteriorhodopsin. A similar interaction is expected in the reconstituted purple membrane.

Discussion

The results presented in this report demonstrate that gramicidin-A does not function as a ionophore in the lipid phase of the membrane under the

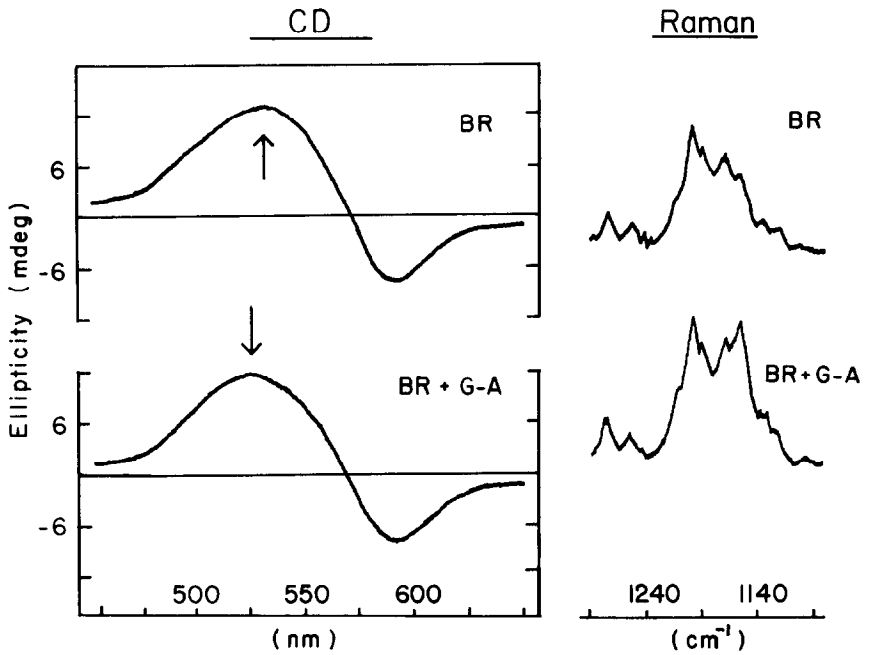

Figure 4. Effects of Gramicidin-A on Spectroscopic Properties of Bacteriorhodopsin in Isolated Purple Membrane. The CD and resonance Raman Spectra of isolated purple membrane in the absence (upper traces) and the presence (lower traces) of 10 μg antibiotic per mg bacteriorhodopin in 150 mM KCl were measured. The CD spectra (left traces) were obtained after repetitive scanning by a micro processor to eliminate random noise. The Raman Spectra (right traces) were obtained by using a rotatory cell to minimize the heating effect of the laser beam. The concentration of bacteriorhodopsin used for the measurements was 0.8 mg/ml.

experimental conditions. Rather, the helical antibiotic interacts with the protein, bacteriorhodopsin, and consequently inhibits the energy transduction process. To determine whether the observed conformational changes of the chromophore is a result of the direct interaction with the retinal group or transmitted from other part, e.g., a helical segment of the protein is currently under investigation.

The data shown in Figure 2 reveals that the ability of reconstituted purple membrane to convert light into transmembranous proton electrochemical potential is completely inhibited by the presence of ∿1.2 gramicidin-A per molecule of bacteriorhodopsin (∿75μg/mg). However, the inhibition reaches 50% with only 1 antibiotic per 4 molecules of the protein (∿15μg/mg). This observation suggests that either there is a positive cooperativity in the process of energy transduction between trimeric units of the protein which

are known to exist in the native membrane (17) or the trimers as well as the monomers which also pump protons (18) but may have different affinity toward the antibiotic, are both present in the reconstituted vesicles.

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