

BRIEF COMMUNICATION

VOLTAGE-DEPENDENT CONDUCTANCE INDUCED BY ALAMETHICIN-PHOSPHOLIPID CONJUGATES IN LIPID BILAYERS

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ABSTRACT Alamethicin, a linear 20-amino acid antibiotic, forms voltage-dependent channels in lipid bilayer membranes. We show here that alamethicin-phospholipid conjugates can be prepared by photolysis of unilamellar vesicles containing alamethicin and a phosphatidylcholine analogue with a carbene precursor at the end of the C-2 fatty acyl chain. This result indicates that at least a portion of the alamethicin molecule is in contact with the hydrocarbon moiety of the membrane in the absence of an applied voltage. Furthermore, the alamethicin-phospholipid photoproduct is able to induce a voltage-gated conductance similar to that of natural alamethicin. The importance of these results in terms of mechanisms for channel gating is discussed.

INTRODUCTION

Several substances obtained from fungi and bacteria form voltage-dependent pores in artificial lipid bilayer membranes (1). One of these substances, alamethicin, has been well characterized with respect to its mode of action in planar lipid bilayers (2-4). Alamethicin is a largely hydrophobic, linear eicosapeptide with an acylated NH_2 -terminus and a COOH -terminal alcohol (5). Because alamethicin is able to induce a voltage-dependent conductance in lipid bilayers similar to that found in nerve and muscle, it has often been used as a model system for studying channel gating and channel formation. Most of the models suggested for alamethicin channel gating propose that the applied electric field leads to the insertion of alamethicin molecules, initially located at the membrane surface, into the hydrocarbon region of the bilayer (6, 7). Here, we report experiments in which alamethicin is photolytically cross-linked to a phosphatidylcholine analogue containing a carbene precursor at the end of the C-2 fatty acyl chain. This result indicates that a portion of the alamethicin molecule is present in the interior of the lipid bilayer in the absence of an applied voltage. The alamethicin-phospholipid photoproduct is able to induce a voltage-gated conductance similar to that of natural alamethicin. We thus suggest that gating is due to conformational changes of the alamethicin located within the bilayer.

METHODS

Alamethicin, obtained from the Upjohn Company (Kalamazoo, Mich.), was acylated with [^3H]acetic anhydride (New England Nuclear, Boston, Mass.; 50 mCi/mmol). The structure given in Fig. 1 *A* for alamethicin [^3H]acetate is that of the main component of natural alamethicin (5, 8). The phosphatidylcholine analogue, 1-palmitoyl-2- ω -(*m*-3,protodiazirinophenoxy) undecanoyl-*sn*-glycerol-3-phosphorylcholine, was synthesized according to Gupta et al. (9). The phospholipid in CHCl_3 was mixed with alamethicin [^3H]acetate in ethanol (molar ratio = 126:1) and dried under nitrogen and vacuum for 20 min. 1 ml of 0.1 M NaCl, 0.1 M Tris-Cl, pH 7.6, was added, and the suspension sonicated under N_2 for 40 min at room temperature. In this condition, the final alamethicin concentration was 4×10^{-6} g/ml. The sonicated vesicles were transferred to a quartz cuvette and photolyzed at 366 nm for 45 s. The half-life of the phenoxy diazine under these experimental conditions is ~ 45 s. The product, after photolysis, was extracted by the Bligh-Dyer method (10). After evaporation by an N_2 stream, the organic residue was taken up in $\text{CH}_3\text{CH}_2\text{OH}:\text{CHOOH}$ (3:1, vol/vol) and separated by Sephadex LH-20 column chromatography. The alamethicin-phospholipid photoproduct represents ~ 25 –30% of the total alamethicin [^3H]acetate. The partially characterized cross-linked photoproduct structure is shown in Fig. 1 *A*.

RESULTS AND DISCUSSION

The steady-state conductance induced by alamethicin [^3H]acetate in lipid bilayer membranes was qualitatively identical to that induced by natural alamethicin, i.e., the steady-state conductance was proportional to the ninth power of the alamethicin [^3H]acetate concentration, and it changed an *e*-fold per 4–5 mV (data not shown). We conclude, therefore, that “normal” gating is not altered by acylation of the COOH-terminal alcohol of alamethicin.

Photochemical irradiation of vesicles containing alamethicin [^3H]acetate and “photoactivable” phospholipids was used to cross-link the alamethicin amino acyl residues present in the bilayer in the absence of an applied voltage (Fig. 1 *A*). Irradiation of the vesicle dispersion at the λ_{max} of the phenoxydiazirine, followed by purification, afforded cross-linked alamethicin [^3H]acetate-phospholipid photoproducts. As shown in Fig. 1 *B*, trifluoroacetic acid hydrolysis of the photoproduct at the proline secondary amide bonds (5) yields peptide fragments 2–20 (I), 14–20 (II), and 2–13 (III). Fragments I and III contain phosphate, whereas none was detected in fragment II. These results locate the site(s) of cross-linking to the NH_2 -terminal segment of alamethicin (as schematically depicted in Fig. 1 *A*). If alamethicin were present in appreciable amounts on the surface of the bilayer vesicles at the time of photolysis, one would have expected appreciable cross-linking to have occurred in the COOH-terminal portion of the eicosapeptide, because the aromatic carbene generated by photolysis can insert (attach) to any amino acid side chain. We did not find any cross-linking near the COOH-terminal. Structural characterization of the photoproduct by amino acid sequencing is in progress.

Fig. 2 *A* shows a steady-state current-voltage curve induced by the photoproduct in a glycerolmonooleate (GMO) membrane. The observed current-voltage curve is asymmetric with respect to the applied voltage. As shown in the inset of Fig. 2 *A*, the photoproduct-induced conductance is an exponential function of voltage. The conductance increases *e*-fold with an increase in voltage of 9.5 ± 1 mV ($n = 5$). We further found that the conductance increases with the third power of the photoproduct concentration ($n = 3$, data not shown). Similar results were obtained in 1,2-diphytanoyl-*sn*-glycerol-3-phosphorylcholine membranes treated with the photoproduct. Thus, the photoproduct induces a voltage-dependent conductance that is less voltage dependent and increases with a smaller power of ionophore

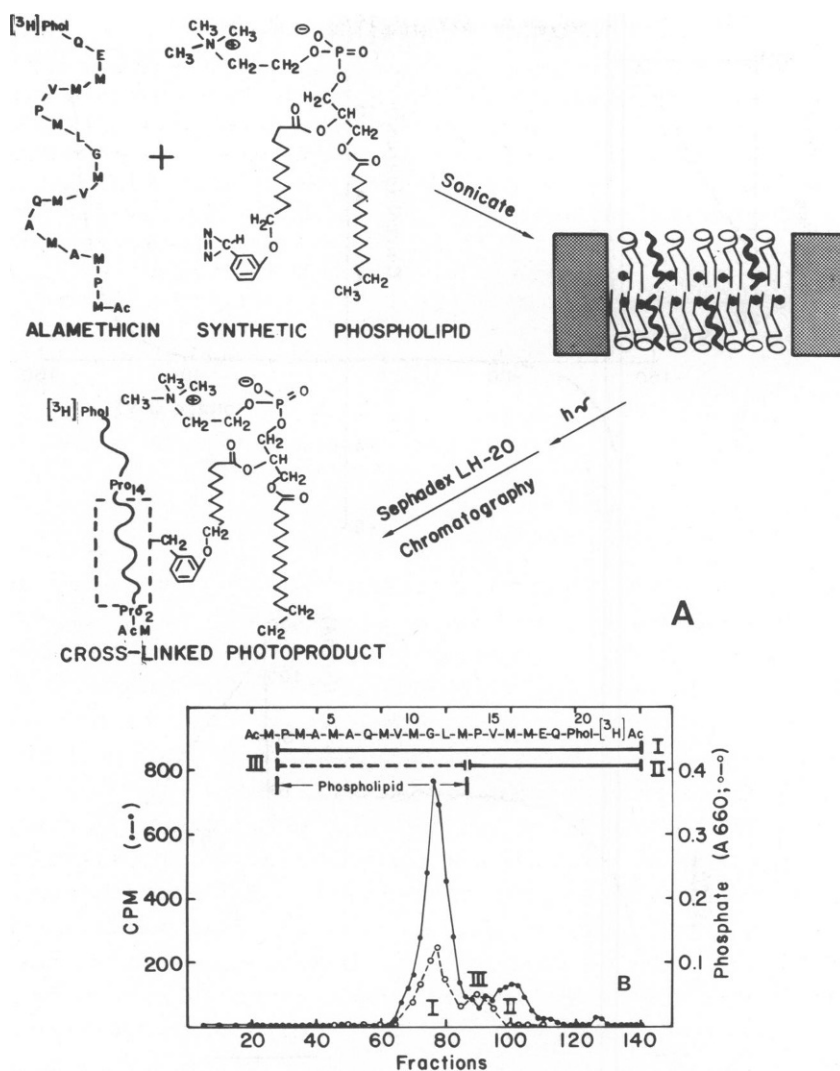


FIGURE 1 (A) Schematic representation of the photochemical cross-linking experiments. For details, see text. (B) The crosslinked alamethicin-phospholipid photoproduct was selectively hydrolyzed by treatment with anhydrous trifluoroacetic acid (TFA) for 24 h at room temperature (5) and then separated on Sephadex LH-20 in $\text{CHCl}_3\text{-CH}_3\text{OH}$ (1:1, vol/vol). The separated fractions were treated with 5.7 N HCl, 110°C for 24 h and the amino acid composition determined with a Beckman amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.). The presence of phosphate in the different fractions was determined by the method of Ames (11). The preliminary structure of alamethicin and the corresponding TFA fragments are shown in the upper part of the figure which was drawn according to Pandey et al. (5). M denotes methyl-alanine (α -amino isobutyric acid), and Phol denotes phenylalaninol.

concentration than alamethicin $[^3\text{H}]\text{acetate}$ or natural alamethicin. Fig. 2 B shows the time-course of the current induced by the photoproduct in response to a 100-mV voltage jump. The current increases monotonically to a new steady state. The time-course of the current is well described by a single first-order relaxation process with a relaxation time of 41 ms (Fig. 2 B, inset). In general, we found that the kinetic properties of the alamethicin-phospholipid

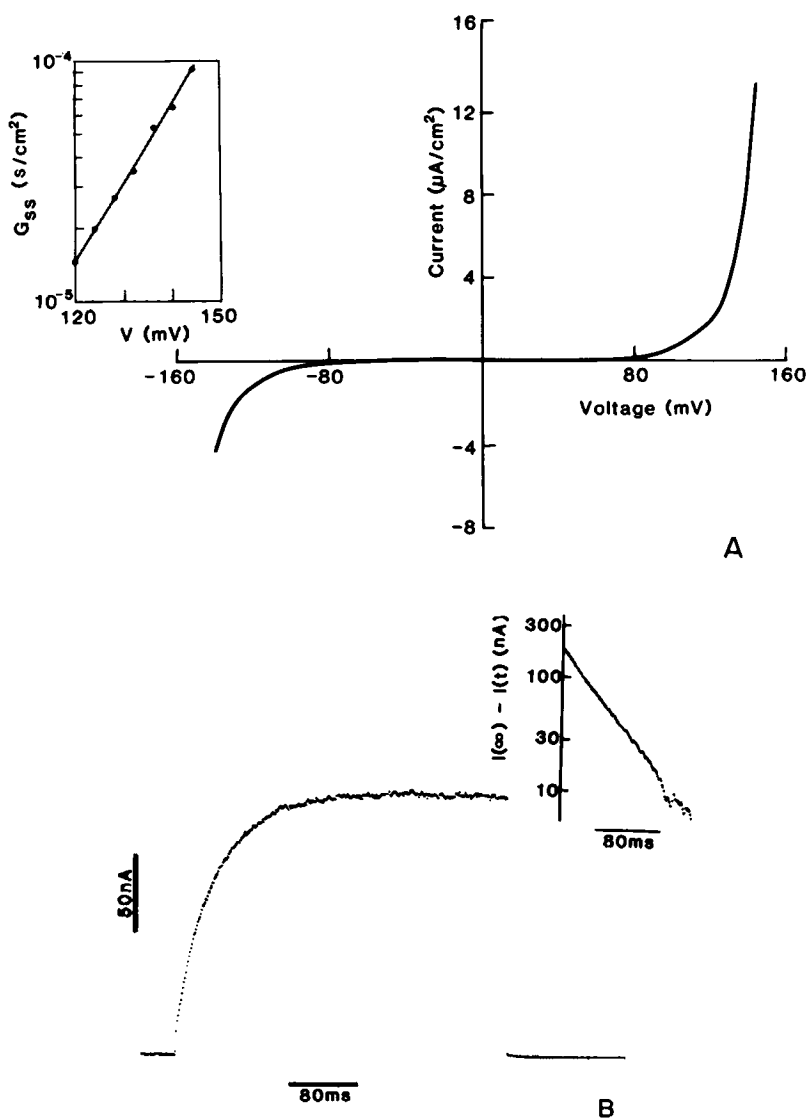


FIGURE 2 (A) Steady-state current-voltage relationship induced by the photoproduct. GMO membranes were formed by apposition of two monolayers as described in detail (12, 13). GMO was obtained from Nu Chek Prep, Inc. (Elysian, Minn.). The aqueous solutions in both compartments consisted of unbuffered (pH 6) 1 M NaCl. The photoproduct was added to only one compartment to a final concentration of 1.3 nM. After addition of the photoproduct, the aqueous phases were stirred for 1 h to ensure equilibrium. Electrical measurements were made using a two-electrode system (13). The side to which the photoproduct was added was defined as the positive side of the membrane, and positive current is defined as the flow of cations away from the photoproduct-containing compartment. All experiments were done at room temperature. The inset shows the steady-state conductance (log scale) as a function of applied voltage in the range of +120 to +150 mV. (B) Time-course of the photoproduct-induced current in response to a voltage jump. At time zero, the voltage was changed from zero to 100 mV. [Photoproduct] = 1.4 nM. Membrane area: $6 \times 10^{-4} \text{ cm}^2$. Other experimental conditions are those of Fig. 2 A. Inset, Semilogarithmic plot of the photoproduct-induced current vs. time from the record of Fig. 2 B. The time-course is well described by a single exponential with a relaxation time of 41 ms and with a regression coefficient of 0.995.

photoproduct at high levels of conductance are very similar to those of natural alamethicin (3, 4, 14).

To examine current fluctuations at low photoproduct concentrations, we took advantage of our observation that the bilayer current relaxation induced by the photoproduct is ~1,000-fold slower in 1,2-diphytanoyl-*sn*-glycerol-3-phosphorylcholine membranes than in GMO membranes under similar experimental conditions. Fig. 3 shows a record of conductance fluctuations arising from a single photoproduct channel in diphytanoyl phosphorylcholine membranes. (The statistical argument for this inference will be discussed in a subsequent report.) The mean lifetime of the channel (~40 s) is similar to the relaxation time of the "on" response in many-channel membranes formed from this lipid (~25 s at 200 mV). Three qualitative characteristics of the single channel formed by the photoproduct are similar to those seen with alamethicin. (a) The channel contains incrementally distinct conductance states. (b) The conductance states are not integral multiples of each other. (c) The mean lifetime of the different conductance states is short compared with the mean lifetime of the open channel. The photoproduct channels can be distinguished quantitatively from those of natural alamethicin in two ways: (a) the photoproduct channel has fewer conductance states (we have found up to four different states; see Fig. 3) than the alamethicin channel (with nine conductance states); (b) the first and second conductance levels of the photoproduct channel are most frequently populated, while the third and fourth levels are the most frequently populated in the alamethicin channel (4, 14).

In summary, we have found that upon irradiation, a photoactivable phospholipid can covalently couple with the NH₂-terminal segment of alamethicin in the absence of an applied electric field. The alamethicin-phospholipid conjugate has a less voltage- and concentration-dependent conductance than natural alamethicin, but is otherwise similar to the parent ionophore. These results suggest that the unit of conductance (channel) induced by the photoproduct is formed by fewer monomers than in the alamethicin channel. The fact that the photoproduct channel has fewer conductance states than the alamethicin channel supports this hypothesis.

These studies indicate that at least the NH₂-terminal end of alamethicin is found within the membrane in the absence of an applied voltage¹ (remembering that cross-linking was done at zero voltage) and that channel gating can be accomplished by alamethicin even when covalently "anchored" within the hydrocarbon region of the bilayer. That alamethicin conjugates are active in channel formation argues that the localization experiments are not merely identifying a population of "incompetent" alamethicin. It is difficult at present to give a quantitative estimate of how deep the NH₂-terminal end goes into the hydrocarbon interior. However, Gupta et al. (16) measured the point of maximum cross-linking of fatty acid acyl chain in phospholipids carrying the carbene precursor at the end of one of the acyl chains. They found that the maximum cross-linking is observed at C₁₂ when the *sn*-2 acyl chain

¹However, it can still be argued that there may be a fraction of monomers randomly flipping in and out of the membrane in the absence of an applied electric field. This process may not originate an ionic conductance, but may lead to the formation of alamethicin-phospholipid conjugates in an amount large enough to explain the present results. This is unlikely, as Boheim and Benz (15) have shown, using the charge-pulse relaxation technique, "no orientation of an appreciable amount of dipole molecules occurs before pore formation." Furthermore, their results indicate that the activation energy for insertion of single monomers is too high and, therefore, the process of insertion will be too slow to explain the present results.

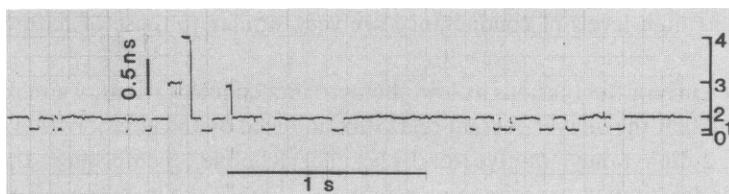


FIGURE 3 Current fluctuations due to a single photoproduct channel in a diphytanoyl phosphatidylcholine membrane. The scale on the right indicates the different conductance levels. In this particular record, four levels of conductance can be seen. [Photoproduct] = 0.1 nM, 1 M NaCl. Applied potential is 180 mV. Channel duration is 40 s, and only a portion of that particular record is shown.

(carrying the carbene precursor) is undecanoyl and the *sn*-1 acyl chain contains 15 carbon atoms. No cross-linking was observed beyond carbon 6. These experiments led us to conclude that (a) if alamethicin were completely located at the membrane surface, as previously suggested (6, 7), it would not be cross-linking with the phospholipid in the absence of applied potential, and (b) the end terminal N of alamethicin is located somewhere between carbon 6 and the center of the bilayer.

Recently, Fringelli and Fringelli (17) used infrared-attenuated total reflection spectroscopy to study the structure of alamethicin in multilamellar states of 1,2-dipalmitoyl-*sn*-glycerol-3-phosphoryl-choline. They concluded that alamethicin is mainly incorporated into the phospholipid hydrocarbon region in the absence of an applied field. Together, these investigations make unlikely a model of alamethicin conductance in which an electrical field-induced rotation of alamethicin monomers from the bilayer surface represents the gating process. The induction of new conformational states of the alamethicin monomers within the lipid bilayer by an applied electric field represents an attractive alternate mechanism for channel gating.

This work was supported by National Institutes of Health grants GM-25277 and GM-28992, and by the Veterans Administration.

Received for publication 22 July 1981 and in revised form 14 September 1981.

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