Gel and liquid-crystalline domains may both be present near the transition temperature of vesicles containing a defined lipid plus protein, especially at higher lipid:protein ratios. Since the line shapes from the gel phase often resemble those of the protein-associated component, an incorrect analysis of the system will lead to an apparent increase in the estimate of bound lipid near and below the transition temperature. Even if the line shapes could be separated, it is clear that the simple equilibrium described by the exchange equation given above no longer applies when the label distribution favors one phase. Measurements of the distribution of 14-proxylstearate as it equilibrates between two populations of liposomes, one gel and the other liquid-crystalline, are shown in Fig. 1 *C*. Starting with labeled liquid-crystalline vesicles (DOPC) and adding gel phase vesicles (DPPC), it is clearly seen that the fatty acid label migrating through the aqueous phase establishes an equilibrium distribution that is 75% in favor of the liquid-crystalline phase. The converse experiment, where the label is trapped in the gel phase before addition of the liquid-crystalline vesicles, shows the same trend, although the system does not reach equilibrium at lower temperatures. At 35°C the end result, however, is the same for both experiments. It is not surprising that lipid packing in the gel phase tends to exclude the lipid spin label (6), since it carries the nitroxide moiety on the acyl chain.

These results illustrate the fact that at low temperatures, especially near or below the lipid transition temperature, spectroscopic line overlap and general thermodynamic and kinetic considerations dictate the need for caution in analyzing lipid-protein interactions. One possible problem that has not turned out to be an obstacle in estimating the equilibrium binding of lipids to protein is the potential perturbation introduced by the spin label. This has been tested (7) and the effects on the equilibrium distribution of lipid binding to several transmembranous proteins are negligible when the temperatures used are well above the transition temperature of the lipid and the nitroxide moiety is near the end of the acyl chain of the lipid spin label (7).

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MOLECULAR MECHANISMS OF ALAMETHICIN CHANNEL GATING

STEVEN C. QUAY
Department of Pathology, Stanford University School of Medicine and the Palo Alto Veterans Administration Hospital, Stanford, California 94305, U.S.A.

RAMON LATORRE
Department of Physiology and Biophysics, Harvard Medical School, Boston, Massachusetts 02115 U.S.A.

Alamethicin is a largely hydrophobic, linear eicosapeptide, with an acylated N-terminus and a C-terminal alcohol (1). 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 field leads to the insertion of alamethicin molecules initially located at the membrane surface into the hydrocarbon region of the bilayer (2). We show here that alamethicin can be 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 moelcule is present in the interior of the 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.

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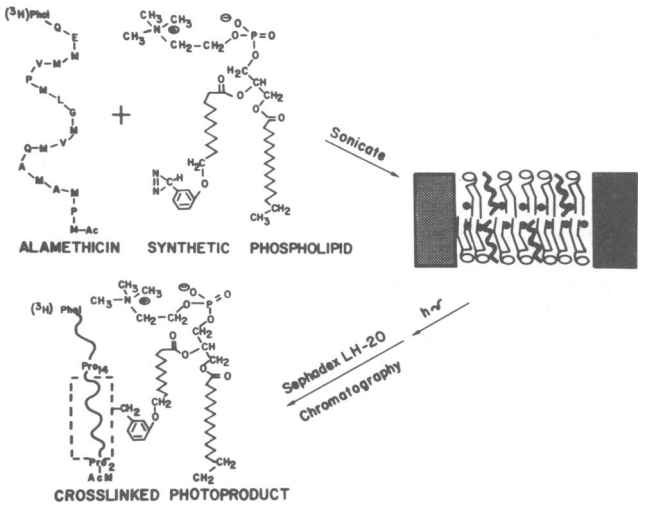

FIGURE 1 Alamethicin was obtained from the Upjohn Company (Kalamazoo, MI) and was acylated with [^{3}H]acetic anhydride (New England Nuclear Co.; 50 mC/mmol). The photoactivable phospholipid, 1-palmitoyl-2-w(m-3, protio-diazirinophenoxy) undecanoyl-*sn*-glycerol-3-phosphorylcholine, was synthesized according to Gupta et al. (5). The phospholipid and alamethicin [^{3}H]acetate (molar ratio=126:1) were sonicated under nitrogen and were photolyzed at 366 nm for 45 s. The half-life of the phenoxydiazirine under these experimental conditions is ~ 4 s. The product, after photolysis, was extracted with chloroform:methanol:water (7:3:1), and separated by Sephadex LH-20 column chromatography. The alamethicin-phospholipid photoproduct represents ~ 25–30% of the total alamethicin [^{3}H]acetate.

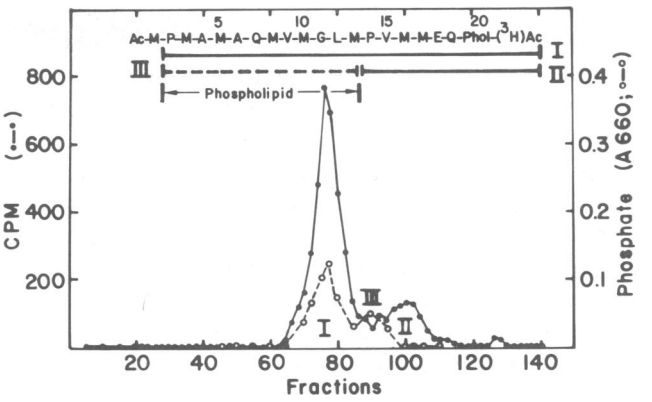

FIGURE 2 The cross-linked alamethicin-phospholipid photoproduct was selectively hydrolyzed by treatment with anhydrous trifluoroacetic acid (TFA) for 24 h at room temperature (1), separated on Sephadex LH-20, and the amino acid and phosphate content of separated fractions determined. The primary structure of alamethicin and the corresponding TFA fragments are shown in the upper part of the figure. M denotes methyl-alanine (α-amino isobutyric acid), and Phol denotes phenylalaninol.

MATERIALS AND METHODS

Photochemical irradiation of vesicles containing alamethicin [^{3}H]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). Normal gating was not altered by acylation of the C-terminus of alamethicin. Irradiation of the vesicle dispersion afforded cross-linked alamethicin [^{3}H]acetate-phospholipid photoproducts. Lipid bilayer membranes were formed by apposition of two monolayers (3). The aqueous solution consisted of unbuffered 1M NaCl and the photoproduct was added to only one side of the membrane.

RESULTS AND DISCUSSION

As shown in Fig. 2, trifluoroacetic acid hydrolysis of the photoproduct at the proline secondary amide bonds yields peptide fragments 2–20 (I), 14–20 (II), and 2–13 (III). Fragments I and III contain phosphate, while none was detected in fragment II. These results locate the site(s) of cross-linking to the N-terminal segment of alamethicin (as schematically depicted in Fig. 1).

The photoproduct is able to induce an alamethicin-like voltage-dependent conductance in glycerolmonooleate (GMO) bilayer membranes. The conductance increases *e*-fold with an increase in voltage of 9.5±1 mV (n=5) and increases with the third power of photoproduct concentration. Similar results were obtained in 1,2-diphytanoyl-sn-glycerol-3-phosphorylcholine (DPC) membranes treated with the photoproduct. Thus, the photoproduct induces a voltage-dependent conductance which is less voltage dependent and increases with a smaller power of ionophore concentration than alamethicin [^{3}H]acetate. We further found that the kinetic properties of the alamethicin-phospholipid photoproduct at high levels of conductance are very similar to those of natural alamethicin. In DPC membranes we found that three characteristics of the single channel formed by the photoproduct are similar to those seen with alamethicin: (*a*) the channel contains incremently 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. However, the photoproduct channel has fewer conductance states (we have found up to four different states) than the alamethicin channel (with nine conductance states) and the first and second conductance states are the most populated. The third and fourth levels are the most frequently populated in the alamethicin channel (2).

In summary, we have found that when irradiated, a photoactivable phospholipid can covalently couple within the N-terminal segment of alamethicin in the absence of an applied 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 and the single channel results suggest that the unit of conductance (channel) induced by the photoproduct is formed by fewer monomers than the alamethicin channel, that the N-terminal portion of alamethicin is found within the membrane in the absence of an applied voltage, and that channel gating can be accomplished by alamethicin even covalently "anchored" within the hydrocarbon region of the bilayer.

Gupta et al. (4) measured the point of maximum cross-linking of fatty acyl chains in phospholipids carrying

the carbene precursor at the end of one of the acyl chains. They found that the maximum cross-linking was observed at C–12 when the *sn*-2-acyl chain (carrying the carbene precursor) is undecanoyl and the *sn*-1 acyl chain contains fifteen carbon atoms. No cross-linking was observed above the C–6 position. These experiments led us to conclude that if alamethicin were completely located at the membrane surface, as previously suggested, it would not be cross-linking with the phospholipid in the absence of an applied potential, and also that the N-terminal end of alamethicin is located somewhere between C–6 and the center of the bilayer. The induction of new conformational states of the alamethicin monomers within the lipid bilayer by an applied electric field represents an attractive alternative mechanism for channel gating.

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