Alamethicin Pyromellitate: An Ion-Activated Channel-Forming Peptide†

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ABSTRACT: The synthesis and characterization of alamethicin pyromellitate (Alm-PM), a derivative of the channel-forming peptide alamethicin bearing three negative charges at the C-terminus, is described. The self-association of Alm-PM in small unilamellar vesicles of dioleoylphosphatidylcholine (DOPC), monitored using circular dichroism (CD) spectroscopy, occurs much less readily than the self-association of unmodified alamethicin. Channel formation by Alm-PM also occurs less readily and exhibits a higher voltage threshold for activation in planar lipid bilayers and in lipid vesicles. An increase in the salt concentration, and particularly the addition of calcium ions, promotes Alm-PM self-association as monitored by CD spectroscopy. Calcium also facilitates channel formation by Alm-PM both in planar lipid bilayers and in lipid vesicles by lowering the voltage threshold for activation. Thus Alm-PM behaves as an ion-activated ion channel. These results indicate that the self-association of alamethicin-like peptides in membranes is critical for channel formation and that transmembrane flip-flop of peptide helices is not required. In addition, these results demonstrate that the activity of channel-forming peptides may be controlled by controlling the process of self-association.

The remarkable ability of the peptide alamethicin to form clearly resolved, voltage-dependent, multistate ion channels has made it a subject of considerable interest for many years (*e.g.*, Latorre and Alvarez (1981) and Sansom (1993a,b)). Alamethicin (R_f 50) is composed of twenty residues and contains no charged groups (Figure 1). It assumes a predominantly helical conformation in membranes (Rizzo et al., 1987; Vogel, 1987; Cascio & Wallace, 1988; Haris & Chapman, 1988; Woolley & Wallace, 1993), and these helices can adopt a transmembrane orientation (Huang & Wu, 1991). The ability of the peptide to form ion channels is generally assumed to depend on the self-association of helical monomers into helix bundles (for reviews, see Latorre and Alvarez (1981), Woolley and Wallace (1992), and Sansom (1993b)). A lumen in the center of such a bundle then provides a pathway for transmembrane ion transport. Details of the process of self-association of alamethicin in membranes are of particular interest both for elucidating the mechanism of alamethicin channel formation (and thereby of a host of related pore-forming molecules (*e.g.*, Molle et al. (1987), Lear et al. (1988), Ojcius and Young (1991), and Sansom (1991))) and for the information they may provide on factors governing the folding and stability of helix-bundle motifs in membrane proteins (*e.g.*, Popot and Engelman (1990)).

Alamethicin

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-
Aib-Pro-Val-Aib-Aib-Gln-Gln-Pheol

Alamethicin-PM

Ac-Aib-Pro-Aib-Ala-Aib-Ala-Gln-Aib-Val-Aib-Gly-Leu-

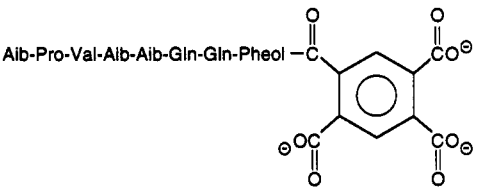

FIGURE 1: Chemical structure of alamethicin (Alm) and alamethicin pyromellityl ester (Alm-PM). The three-letter codes for standard amino acids are used. Aib stands for α-aminoisobutyric acid, and Pheol is phenylalaninol.

We have shown previously that alamethicin self-association can be monitored using circular dichroism (CD)[1] spectroscopy when the peptide is bound to small unilamellar lipid vesicles (Cascio & Wallace, 1988; Woolley & Wallace, 1993). To probe further this process of self-association in membranes and to examine its relationship to channel formation by alamethicin, we have attempted to influence the process by adding charged groups that could modulate the degree of electrostatic repulsion between alamethicin monomers. We report here the synthesis, propensity for self-association

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[1] Abbreviations: Alm, alamethicin; Alm-PM, alamethicin pyromellitate; BES, 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid; CD, circular dichroism; DMAP, 4-(dimethylamino)pyridine; DMF, dimethylformamide; DMSO, dimethyl sulfoxide; DOPC, dioleoylphosphatidylcholine; DPhyPC, diphytanoylphosphatidylcholine; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; IR, infrared; *I/V*, current/voltage; Pheol, phenylalaninol; SUV, small unilamellar vesicle; VAL, valinomycin.

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(measured by CD spectroscopy), and channel-forming ability (measured in a vesicular system and by using the planar bilayer technique) of a pyromellitic derivative of alamethicin: alamethicin-PM (Figure 1). This derivative self-associates in membranes in a manner that is very dependent on the ionic strength in the surrounding solution. Channel formation by alamethicin-PM (Alm-PM) is also observed to be ionic strength dependent and is greatly facilitated by millimolar concentrations of divalent (*e.g.*, Ca^{2+}) ions in the medium.

These results directly correlate the self-interaction of alamethicin-like peptides with their channel-forming properties. While consistent with several proposed models for alamethicin channel formation, the observation of channel formation by this highly charged alamethicin derivative is difficult to reconcile with those models in which flip-flop of peptides across the membrane is an obligatory step (Boheim et al., 1983). These results further suggest that peptide-based ion channels responsive to specific signals (*e.g.*, an elevated Ca^{2+} concentration) might be successfully designed and engineered by controlling the process of self-association in membranes.

MATERIALS AND METHODS

Alamethicin was obtained from Sigma Chemical Co. (St. Louis, MO). This is the R_f 50 version of alamethicin with Gln at position 18 (Figure 1). The sequence is heterogeneous with respect to position 6; Ala or Aib (α-aminoisobutryic acid) occur in approximately equal proportions, as confirmed using HPLC and FAB-mass spectrometry. Peptide solutions were prepared gravimetrically, and the concentrations were checked using a value for the mean residue ellipticity in methanol at 220 nm of −12 500 deg cm^2 $dmol^{-1}$ (Schwarz et al., 1987). Dioleoylphosphatidylcholine (DOPC) and diphytanoylphosphatidylcholine (DPhyPC) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). Pyromellitic anhydride and 4-(dimethylamino)pyridine (DMAP) were obtained from Aldrich Chemical Co. (Milwaukee, WI). The gel filtration medium, Sephadex LH-20, was obtained from Sigma (St. Louis, MO). All other reagents and solvents were of the highest grade commonly available.

Synthesis of Alamethicin Pyromellitate (Alm-PM). Alamethicin (7.55 mg dried over phosphorous pentoxide) was dissolved in 0.2 mL of dry dimethylformamide (DMF). To this solution was added 0.5 mL of pyridine followed by 0.3 mL of a solution (200 mg/mL) of pyromellitic anhydride in DMF and 0.1 mL of a solution (100 mg/mL) of DMAP in DMF. This mixture was stirred at room temperature overnight, whereupon 1.1 mL of distilled water was added in 100-μL increments. After being stirred for 10 min, this solution was then applied to a gel filtration column (LH-20, 60 × 1.5 cm) running in methanol. Absorbance of the eluate was monitored at 280 nm; the first peak to be eluted was pooled, and the methanol was evaporated. The peptide was further purified by passage through a short reverse-phase cartridge (Sep-Pak C_{18}, Millipore, Milford, MA). The cartridge was first washed with 3 mL of acetonitrile followed by 6 mL of distilled water. The Alm-PM sample from the LH-20 column was dissolved in 2 mL of 10% acetonitrile in water. A 0.5-mL aliquot of this solution was then applied to the C_{18} cartridge, which was then washed with 5 mL of distilled water followed by 6 mL of acetonitrile. This procedure was repeated with a fresh cartridge for each 0.5-mL sample of the peptide solution. The acetonitrile fractions were combined and concentrated. A small volume of distilled water (1–2 mL) was added, and then the remainder of the acetonitrile was evaporated. The aqueous peptide solution was then lyophilized, giving a white fluffy solid which was stable for several months at −20 °C. Thin-layer chromatography (chloroform/methanol/water, 65/25/4) of Alm-PM gave a single spot (R_f 0.1) indicating a compound significantly more polar than the starting material (Alm-Gln^{18}), which gave R_f 0.39 in the same system. Thin-layer chromatography was used to regularly check the preparation for hydrolysis over time. Fast atom bombardment mass spectrometry (FAB-MS) was performed at the Carbohydrate Research Centre (University of Toronto) using a VG analytical ZAB SE mass spectrometer. Alm-PM gave a molecular ion (MNa^+) peak at 2222 Da, confirming the pyromellityl modification. The fragmentation pattern seen in the mass spectrum confirmed that the modification had occurred on the C-terminal hydroxyl group of alamethicin.

CD Experiments. For experiments with lipid vesicles, aliquots of stock solutions of Alm or Alm-PM (0.5 mM in HPLC grade methanol) were initially added to a 5 mM sodium phosphate buffer solution (pH 7.0). Methanol concentrations in the samples for CD analysis never exceeded 5%, and equivalent concentrations of methanol were added to control samples. The CD spectrum of alamethicin in water is hardly affected by additions of methanol up to almost 20%. Stock solutions of small unilamellar vesicles (SUVs) at known concentrations were made routinely by sonication of lipid suspensions as described previously (Cascio & Wallace, 1988; Woolley & Deber, 1988). Aliquots of these stock solutions were then added to the alamethicin samples to obtain final lipid concentrations as indicated in the figure captions. No time dependence was observed for any of the reported CD spectra except when calcium was added to high concentrations of Alm-PM in lipid vesicles. In this case a time-dependent macroscopic aggregation occurred.

Experiments in which the salt (KCl) concentration was varied were performed in two ways. Either an aliquot of a concentrated salt solution was added directly to the sample or samples prepared in high-salt solutions were mixed with samples prepared in low-salt solutions. In either case, after mixing, the samples were sonicated briefly in a bath sonicator prior to CD measurement in order to dissipate any ion gradients created across the vesicle membranes.

CD measurements were made in quartz cells (1–0.01-cm path lengths) using AVIV 62DS and 60DS spectropolarimeters. The measurement of optical rotation was calibrated using (+)-10-camphorsulfonic acid, and the wavelength scale was calibrated using benzene vapor. Data was imported into a graphical spreadsheet (Deltagraph, Deltapoint, Inc.) where a correction for baseline drift at 260 nm was made and the scans were averaged and smoothed. Further details of specific experimental conditions are found in the figure captions.

Fluorescence Measurements with Lipid Vesicles. A vesicle system having a transmembrane diffusion potential was prepared as described previously (Woolley et al., 1987). Briefly, lipid vesicles were prepared by sonication as for the CD experiments except that a high lipid concentration was used (100 mg/mL) and the buffer was 200 mM KCl and 5 mM sodium phosphate, pH 7.0. Vesicles were diluted 200-fold into isotonic sucrose medium (335 mM sucrose and 5 mM sodium phosphate, pH 7.0) so that an ion gradient was created across the membrane. Addition of the potassium-specific ionophore valinomycin (0.16 μM in DMSO) leads to the formation of a diffusion potential across the vesicle membranes. The size of this potential may be calculated from the Nernst

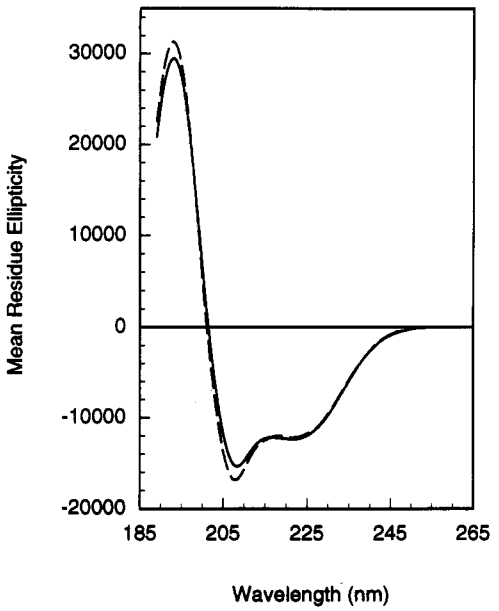

FIGURE 2: CD spectra of Alm (solid line) and Alm-PM (dashed line) at a concentration of 100 μM in methanol.

equation:

$$\Delta\Psi = RT/zF \ln([K^+]_{out}/[K^+]_{in})$$

$[K^+]_{out}$ = 1 mM; $[K^+]_{in}$ = 200 mM;

RT/zF = 25.3 mV @ 20 °C

$$\Delta\Psi = 134 \text{ mV} \quad \text{(negative inside)}$$

Membrane potential was monitored using the potential-sensitive fluorescent dye safranine O (1 μM). The fluorescence emission of this dye varies in a sigmoidal manner with membrane potential (Woolley et al., 1987) but shows an approximately linear response between 150 and 50 mV under these conditions. Fluorescence emission was monitored at 581 nm (excitation was at 522 nm) using an SLM-Aminco series 2 luminescence spectrometer. Peptide solutions were added in methanol to the final concentrations indicated in the figure captions; methanol alone had no effect. Calcium was added from a 1 M $CaCl_2$ stock solution in water.

Planar Bilayer Measurements. Planar bilayers were formed using the Montal–Mueller technique (Montal & Mueller, 1972) across a hole (~100-μm diameter) in a Teflon film sandwiched between Teflon blocks containing 2-mL reservoirs. The hole was pretreated with hexadecane, and bilayers were formed from diphytanoylphosphatidylcholine (DPhyPC). Aliquots of Alm or Alm-PM (0.1 mg/mL in methanol) were added to one side only (designated the *cis* side) to the final concentrations indicated in the figure captions. Currents were measured using an Axopatch-1D amplifier with a CV-4B headstage and filtered at 2 KHz prior to storage on a Sony PCM-VCR. Currents were resampled at 5 kHz on playback and analyzed using ASYST. Solutions were either 0.5 or 4 M KCl with 10 mM BES buffer, pH 7.0. Calcium was added from a 1 M stock solution in water to both sides of the membrane.

RESULTS

Circular Dichroism Studies. The CD spectra of Alm and Alm-PM when dissolved in methanol are closely similar (Figure 2). Upon interaction with small unilamellar vesicles, however, distinct differences are observed. Figure 3 shows

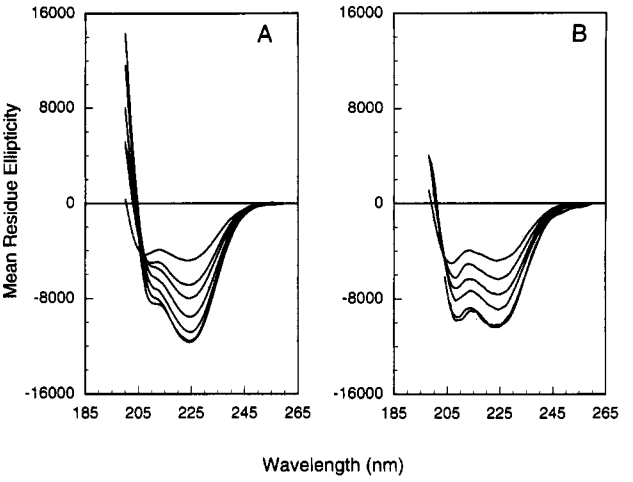

FIGURE 3: Titration of aqueous solutions of Alm (A) and Alm-PM (B) with DOPC lipid vesicles. The peptide concentration in each case was 50 μM in 5 mM sodium phosphate buffer, pH 7.0. The upper curve in each panel corresponds to peptide before the addition of vesicles. Progressively lower curves correspond to progressively larger concentrations of added DOPC as follows: For Alm (A), DOPC concentrations are 0, 0.32, 0.63, 0.127, 0.433, 0.947, and 3.05 mM; for Alm-PM (B), DOPC concentrations are 0, 0.947, 1.86, 3.05, 5.82, and 8.36 mM. Equivalent spectra of samples without peptide were subtracted to correct for lipid contributions to the spectra.

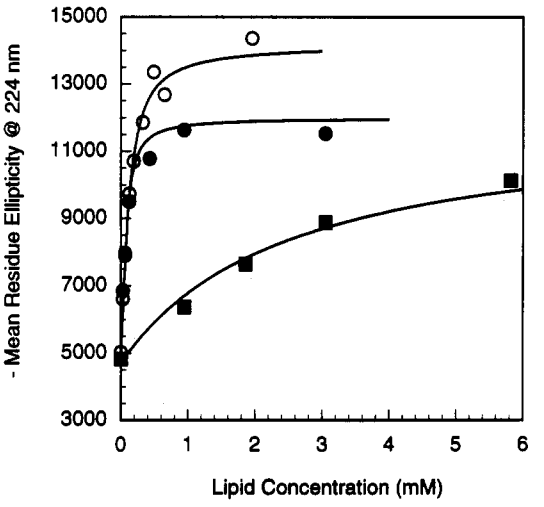

FIGURE 4: Binding curves for Alm (●), Alm-PM (■), and Alm-PM in the presence of 1M KCl (○). Peptides were at a concentration of 50 μM in 5 mM sodium phosphate, pH 7.0. Mean residue ellipticity (×(-1)) at 224 nm (deg cm^2 dmol^{-1}) is plotted versus added DOPC concentration.

the effect of increasing amounts of DOPC vesicles on the peptide CD spectra. When present in aqueous solution in the absence of vesicles, each peptide gives a CD signal indicative of some helical secondary structure (the uppermost curves in panels A and B of Figure 3). As lipid vesicles are added and the peptides bind lipid, the helix content increases, eventually reaching a maximum. The concentration of lipid required to achieve the maximum is considerably higher in the Alm-PM case than with unmodified alamethicin. This is evident from a plot of the change in the CD signal at 224 nm versus the lipid concentration (Figure 4). It is not only the affinity for lipid that is different, however. The *shapes* of the CD spectra are dissimilar. In the fully bound CD spectrum of Alm the minimum at 224 nm is much more pronounced than the minimum at 210 nm, whereas in the Alm-PM spectrum the minima are of about equal intensity (lowermost curves in Figure 3, panels A and B).

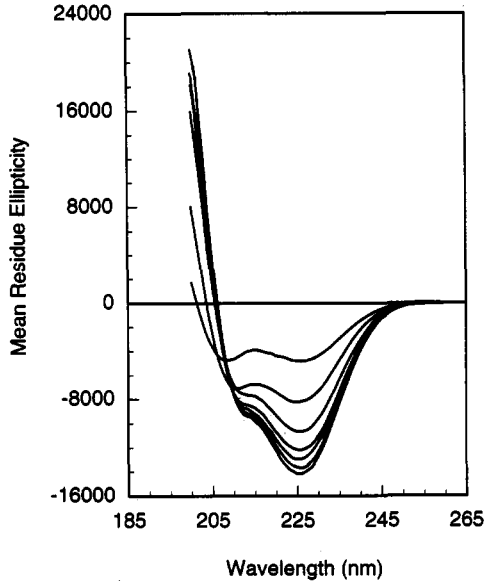

FIGURE 5: Effects of added salt on the CD spectrum of Alm-PM. The uppermost curve is the CD spectrum of Alm-PM (170 μM) in 5 mM sodium phosphate buffer, pH 7.0. The next lower curve is the CD spectrum with the addition of 1.8 mM DOPC. Further curves are with the progressive addition of KCl at concentrations of 12, 164, 266, 455, and 626 mM.

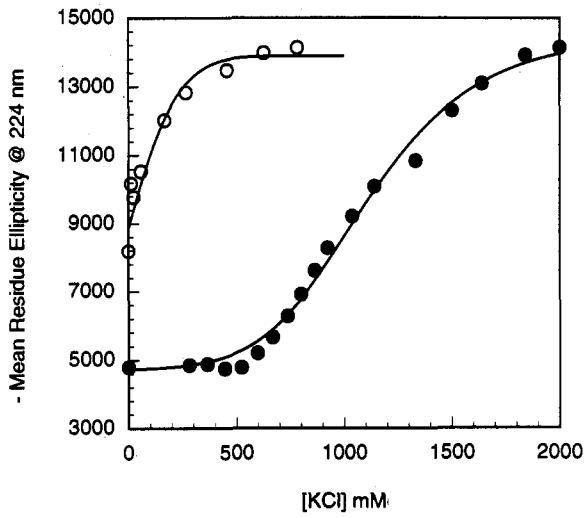

FIGURE 6: Effect of salt on the mean residue ellipticity of Alm-PM in DOPC vesicles at two different peptide concentrations. Mean residue ellipticity (×(−1)) at 224 nm is plotted versus KCl concentration (mM) for 170 μM Alm-PM in 1.8 mM DOPC (○) and for 12 μM Alm-PM in 0.4 mM DOPC (●).

The effect of varying ionic strength was then tested. The titration of Alm-PM with DOPC vesicles was repeated in the presence of 1 M KCl. The apparent affinity of Alm-PM for lipid was then comparable to that of unmodified alamethicin at low ionic strength (Figure 4, open circles). Figure 5 shows the effect of adding progressively higher concentrations of KCl to a solution of Alm-PM and DOPC vesicles. Clearly, as the salt concentration increases, the shape of the CD spectrum changes to resemble that of unmodified Alm bound to DOPC.

The ionic strength required to effect changes in the CD spectrum of Alm-PM in DOPC membranes depends on the peptide concentration. This is shown in Figure 6 where the mean residue ellipticity at 224 nm is plotted as a function of KCl concentration for two different peptide concentrations. At lower peptide concentrations considerably higher ionic strengths are required to effect conversion. For instance, at

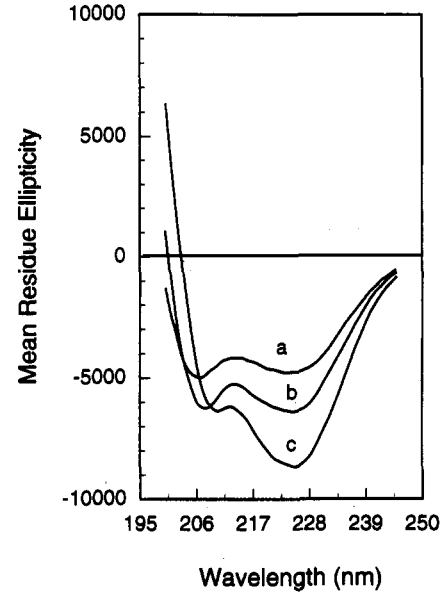

FIGURE 7: Effect of calcium on the CD spectrum of Alm-PM in DOPC vesicles. The uppermost curve (a) is Alm-PM (50 μM) in 5 mM phosphate buffer, pH 7.0. The next lower curve (b) is with the addition of 0.95 mM DOPC. The lowermost curve (c) is with the further addition of 1 mM $CaCl_2$.

170 μM Alm-PM the conversion is half-maximal at 100 mM KCl, whereas with 12 μM Alm-PM conversion is half-maximal at a concentration near 1 M KCl.

Importantly, the addition of a *divalent* cation (Ca^{2+}) at a much lower concentration (1 mM for a peptide concentration of 50 μM) can also promote the CD transition of Alm-PM (Figure 7). A detailed CD study of the effects of calcium over a range of peptide and lipid concentrations is not possible since, at high peptide concentrations, a time dependence in the CD spectra was observed over the course of approximately 0.5 h. Immediately after the addition of calcium, the spectrum reported in Figure 7 was observed. After some time, however, spectra characteristic of highly scattering samples were observed and the solutions became visually turbid. These spectra could be readily distinguished from the spectrum reported in Figure 7 by their greatly diminished CD intensity.

Channel Formation in Lipid Vesicles. The comparative ability of Alm and Alm-PM to dissipate diffusion potentials created across lipid vesicle membranes was then examined. Figure 8 shows changes in fluorescence of the potential-sensitive fluorescent dye safranine O versus time. Vesicle samples were prepared with an internal KCl concentration of 200 mM and an external KCl concentration of 1 mM. In panel A the first arrow indicates the addition of the potassium-specific ionophore valinomycin. This addition results in an increase in dye fluorescence with time corresponding to the charging of the vesicular membranes (Woolley et al., 1987). The second arrow indicates the addition of peptide (Alm or Alm-PM). Unmodified Alm (3.3 μM) caused a drop in fluorescence (to approximately that observed before valinomycin addition) indicating a nearly complete dissipation of the membrane potential. In contrast, Alm-PM at the same concentration (3.3 μM) caused a much less significant drop. Figure 8 (panel B) shows the effect of added calcium. The addition of 3.3 μM Alm-PM (second arrow) caused partial dissipation as before. A 10-fold lower concentration of unmodified Alm (0.33 μM) had to be added to see a comparable effect (panel B, second arrow). Subsequent addition of 2 mM calcium (as $CaCl_2$) (third arrow) had no effect on the system when only Alm was present but caused

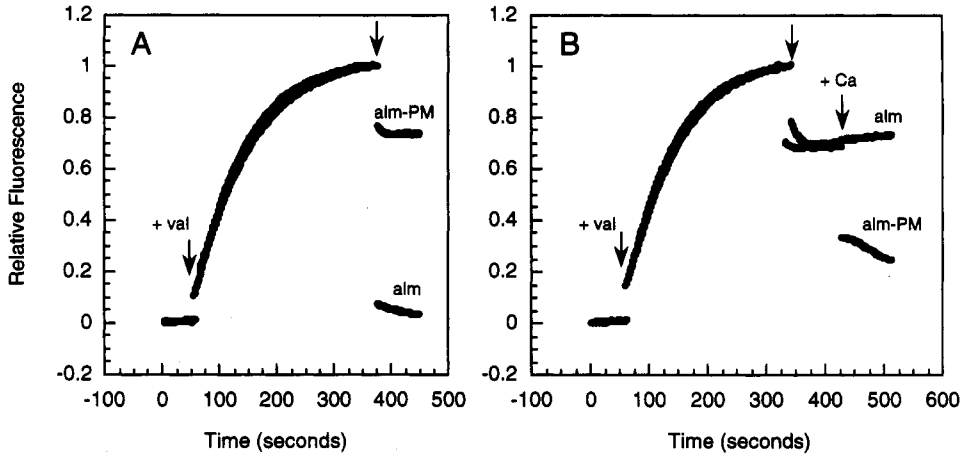

FIGURE 8: Potential-dependent behavior of Alm and Alm-PM in a lipid vesicle system. Lipid vesicles (0.64 mM DOPC, 200 mM KCl, and 5 mM sodium phosphate, pH 7.0 inside) were mixed with 1 μM safranine O in 355 mM sucrose and 5 mM sodium phosphate, pH 7.0. Addition of valinomycin (first arrow, + val) causes a rise in the emission intensity of safranine O fluorescence. In panel A addition of 3.3 μM Alm (second arrow) causes an abrupt decrease to near baseline fluorescence (curve marked Alm). Addition of 3.3 μM Alm-PM instead causes a much smaller decrease in fluorescence (curved marked Alm-PM). In panel B addition of valinomycin causes a fluorescence increase as before (first arrow, + val). Addition of Alm-PM (second arrow) to a concentration of 3.3 μM causes a decrease in fluorescence as seen in panel A. Addition of 0.33 μM Alm has a comparable effect. The subsequent addition of 2 mM $CaCl_2$ (third arrow) has no effect on the sample containing Alm (curve marked Alm), whereas with Alm-PM it causes a further significant drop in fluorescence (curved marked Alm-PM).

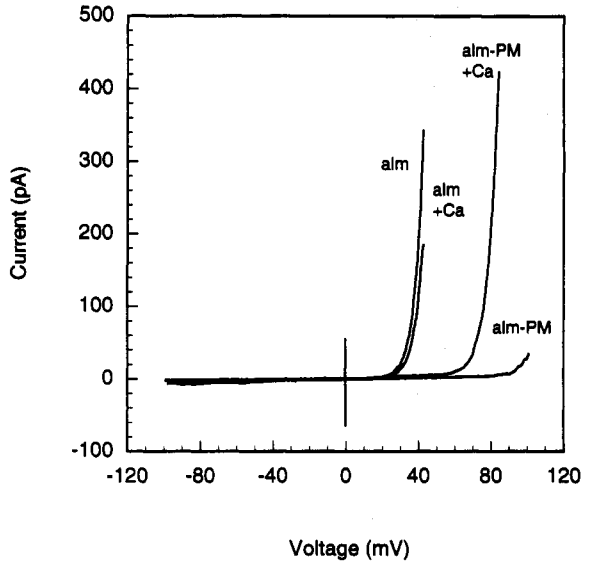

FIGURE 9: Current–voltage (I/V) curves measured in the planar lipid bilayer system. Alm or Alm-PM was added to a concentration of 0.15 μM to one side of the membrane only. The buffer was 0.5 M KCl and 10 mM BES, pH 7.0. Triangular voltage ramps were applied with a period of about 60 s, and the current was recorded. About 50 such ramps were averaged to obtain the curves shown here. Voltage is referenced with respect to the side of the membrane to which the peptides are added (*i.e.*, a positive voltage means the peptide-containing side is made positive). The effect of adding 50 mM $CaCl_2$ to both sides of the membrane on the Alm I/V curve (Alm + Ca) and the Alm-PM I/V curve (Alm-PM + Ca) is shown.

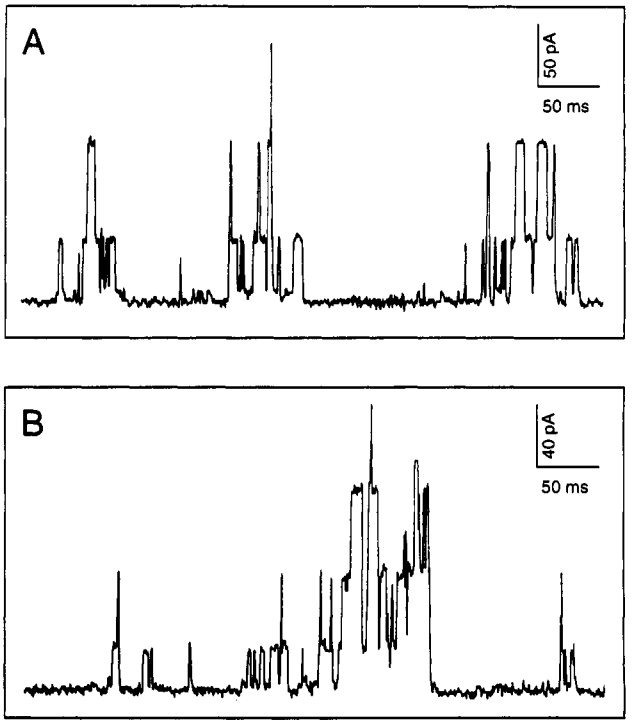

FIGURE 10: Sections of single-channel recordings for Alm-PM (0.15 μM) before (panel A) and after (panel B) the addition of 50 mM $CaCl_2$. The buffer was 0.5 M KCl and 10 mM BES, pH 7.0. The applied voltage was +100 mV (panel A) and +70 mV (panel B).

a further significant dissipation of potential in the presence of Alm-PM.

Planar Bilayer Experiments. To characterize further the behavior of Alm-PM at the single-channel level and in the absence of any other membrane-associated compounds (valinomycin or safranine O), we examined its behavior in planar lipid bilayers. Alm or Alm-PM, at a concentration of 0.15 μM, was added to one side only (designated the *cis* side) of a planar bilayer of diphytanoylphosphatidylcholine. Figure 9 shows current–voltage relations obtained in the presence and absence of added calcium (50 mM) in a background of 0.5 M KCl. In the absence of added calcium Alm-PM is much less effective at forming channels than unmodified Alm. Whereas the threshold voltage (V_t, defined here as the voltage required for an average conductance of 1 nS) for Alm is +30 mV, V_t for Alm-PM under identical conditions is +105 mV. The addition of 50 mM Ca^{2+} has very little effect on Alm. With Alm-PM, however, a shift in V_t to +75 mV is seen—a decrease of about 30 mV. The characteristics of single channels formed by Alm-PM do not appear to be greatly altered by the addition of calcium (Figure 10; Table 1).

Channel formation by Alm-PM is observed readily at very high (4 M) KCl concentrations. Figure 11 shows segments of a single-channel recording of Alm-PM in 4 M KCl and unmodified Alm under equivalent conditions. Although the (multistate) conductances of the two peptides are of com-

Table 1: Effect of Calcium on Subconductance Levels of Alm-PM[a]

conductance (nS)	Alm-PM (0.5 M KCl)	Alm-PM + Ca^{2+} (0.5 M KCl + 50 mM $CaCl_2$)
level 1	(0.04)	(0.08)
level 2	0.45	0.32
level 3	1.1	0.96
level 4	1.9	1.7

[a] Calculated by fitting Gaussian curves to conductance histograms of single-channel data.

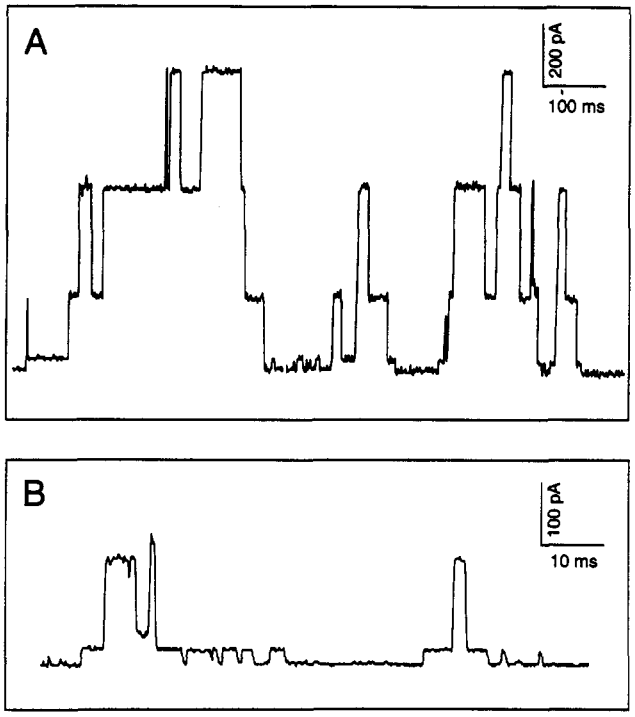

FIGURE 11: Sections of single-channel recordings for Alm-PM (0.15 μM) (Panel A) and Alm (0.015 μM) (Panel B) in 4 M KCl and 10 mM BES, pH 7.0. The applied voltage was +80 mV (panel A) and +60 mV (panel B).

Table 2: Subconductance Levels of Alm and Alm-PM in 4 M KCl[a]

conductance (nS)	Alm (4 M KCl)	Alm-PM (4 M KCl)
level 1	0.3	0.4
×2	0.63	
level 2	2.4	2.8
level 3		6.9
level 4		11.2

[a] Calculated by fitting Gaussian curves to conductance histograms of single-channel data.

parable magnitude (Table 2, Figure 12), the state lifetimes are considerably longer for Alm-PM than for Alm (note the difference in time scales (Figure 11)). Furthermore, unmodified Alm occurs most commonly in the first and second subconductance states, whereas Alm-PM occurs commonly in four or five different subconductance states (Figure 12; Table 2).

DISCUSSION

A general scheme outlining the interactions between alamethicin and lipid membranes is presented in Figure 13 (Panel A). Peptides may occur free in solution or membrane-bound either as monomers or in a self-associated state. Membrane binding causes CD spectral changes characteristic of an increase in helical secondary structure (Rizzo et al.,

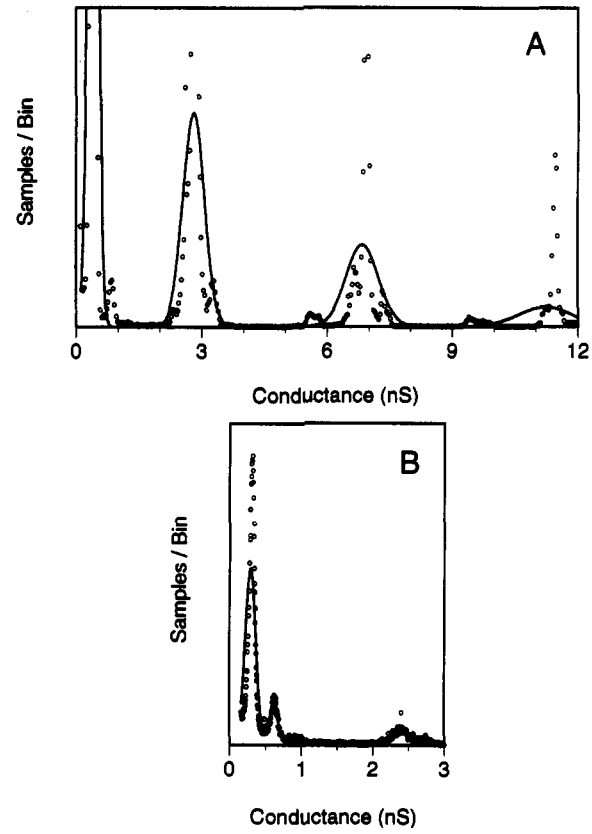

FIGURE 12: Histograms showing the relative frequency of occurrence of different subconductance levels for Alm-PM (A) and Alm (B) in 4 M KCl. Single-channel data (like that shown in Figure 11) was digitized and binned. Small peaks such as the one at 0.63 nS in panel B correspond to the presence of two separate channels, each open in the first subconductance level.

1987; Woolley & Wallace, 1993), indicated by an increase in the length of the rectangles in the diagram. We have shown previously that self-association involves a further characteristic change in the CD spectrum of the peptide (Woolley & Wallace, 1993). Ion-conducting channels are presumed to arise from these self-associated states either through voltage-driven structural changes (*e.g.*, Fox and Richards (1982), Boheim et al. (1983), and Hall et al. (1983)) or spontaneously for certain types of aggregate (*e.g.*, Baumann and Mueller (1974) and Schwarz et al. (1986)). While channels are likely to be aggregates of transmembrane helices, other sorts of aggregates could also exist with a variety of conformations and topologies. Similarly, although there is evidence that monomeric alamethicin in membranes is predominantly surface-associated (Huang & Wu, 1991), a dynamic ensemble of monomer conformations and topologies presumably also exists (Quay & Latorre, 1981; Vogel, 1987).

To understand better the relationship between the self-association of alamethicin and channel formation, we wished to synthesize a highly charged derivative of the peptide in an attempt to modulate the degree of self-association. Alamethicin R_f50 contains no free amino or carboxyl groups for straightforward derivatization. The C-terminal alcohol (Pheol), however, provides a site for acylation. In the presence of the highly active acylation catalyst 4-(dimethylamino)pyridine, alamethicin reacts with pyromellitic anhydride to give the derivative Alm-PM (Figure 1). Pyromellityl derivatives of gramicidin (Apell et al., 1977) and monensin (Fuhrhop & Liman, 1984) have been reported. At neutral pH, Alm-PM is expected to be negatively charged; the pK_a values of trimellitic acid ($C_6H_3(CO_2H)_3$) are 2.5, 3.8, and 5.2 (Maxwell

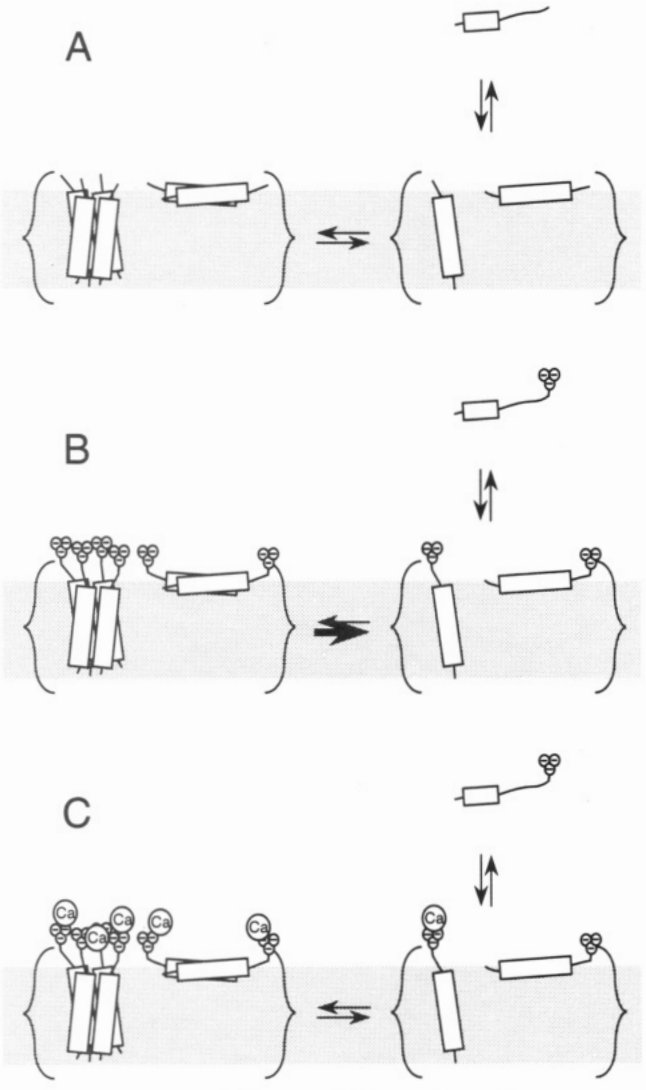

FIGURE 13: Schematic diagram of the membrane interactions of Alm (A), Alm-PM (B), and Alm-PM in the presence of calcium (C). In each case three distinct populations of peptide are identified: (1) peptide free in solution, (2) monomeric membrane-bound peptide, and (3) self-associated membrane-bound peptide. For a discussion, see text.

& Partington, 1937).[2] The charge is located at the extreme C-terminal end of the peptide and so could remain at the membrane surface if the peptide assumed a transmembrane orientation.

Alamethicin Pyromellitate Interactions in Membranes. In methanol, Alm and Alm-PM are monomeric (Cascio & Wallace, 1988; Archer et al., 1991; G. A. Woolley, unpublished). The close similarity of the CD spectra of the two peptides reported in Figure 2 indicates that the C-terminal modification does not alter the conformational properties of the peptide in this solvent. This result also indicates that contributions to the CD signal in this region by the pyromellityl group itself are minimal.

In aqueous solutions in the absence of lipid, Alm and Alm-PM again give similar CD spectra (Figure 3, top curves). However, as small unilamellar vesicles (SUVs) of dioleoylphosphatidylcholine (DOPC) are added, distinct differences arise: (1) The shape of the CD spectrum at high lipid concentrations is different for Alm and Alm-PM. (2) For the same peptide concentration considerably more lipid has to be added to Alm-PM to cause a maximal change in the CD spectrum. Although artifacts may arise in the CD spectra of membrane systems as a result of absorption flattening and differential scattering (Gordon & Holzwarth, 1971; Mao & Wallace, 1984; Glaeser & Jap, 1985), we have presented detailed evidence elsewhere that for this system under these conditions such effects are minimal (Woolley & Wallace, 1993).

The most distinct difference in the fully bound CD spectra of Alm and Alm-PM is that in the Alm spectrum the minimum at 224 nm is much more pronounced than the minimum at 210 nm, whereas in the Alm-PM case the minima are of about equal intensity. Importantly, a CD spectrum exactly like the Alm-PM spectrum is observed with Alm itself at low concentrations (4 μM) in DOPC vesicles (Woolley & Wallace, 1993). The obvious conclusion, therefore, is that Alm-PM is monomeric in DOPC vesicles under these conditions. This is presumably a result of electrostatic repulsion between the highly negative C-termini of Alm-PM monomers.

In the scheme depicted in Figure 13 (panel B) we have indicated that the dynamic equilibrium of membrane-associated Alm-PM peptides is shifted toward monomeric forms when compared to the unmodified Alm case (panel A). While this shift alone should decrease the apparent affinity of Alm-PM for membranes (Figure 4), there might also be changes in the intrinsic affinity of monomeric peptides for membranes.

The observed effects of ionic strength on the CD spectra of Alm-PM support the conclusion that Alm-PM is monomeric because of electrostatic repulsion. Increasing concentrations of KCl, which would be expected to screen the negative charge of the pyromellityl moiety, cause a transition in the CD spectrum characteristic of self-association (Figure 5). In addition, high ionic strength results in an increase in the apparent affinity of Alm-PM for DOPC vesicles (Figure 4, open circles). Changes in the apparent affinity will reflect any salt effects on the energetics of monomer association with membranes as well as effects on the energetics of self-association within membranes. Salt effects on self-association are definitely important, however, since at low ionic strength Alm-PM is monomeric even when present at high concentrations in membranes (Figure 5).

The salt concentration required to effect the self-association of Alm-PM depends on the peptide concentration. As the peptide concentration decreases, more KCl is required (Figure 6). Interestingly, the intensity of the CD signal measured for Alm-PM at high KCl concentrations is greater than that measured for Alm at low salt (Figures 5 and 6), although the shapes are the same. This might indicate some difference in the degree of self-association between the two systems or small differences in peptide helical content. High salt concentrations have also been reported to increase the CD intensity of unmodified Alm bound to DOPC vesicles (Rizzo et al., 1987).

The effectiveness of added salt in promoting self-association depends on the nature of the salt. The addition of a divalent cation (Ca^{2+} as $CaCl_2$) can promote self-association at much lower total salt concentrations. This effect of calcium is shown schematically in Figure 13 (panel C). Calcium is proposed to screen the electrostatic repulsion between Alm-PM monomers and thereby promote the occurrence of self-associated forms of the peptide.

Channel-Forming Ability. To probe the functional consequences of the different propensities toward self-association of Alm and Alm-PM, we examined their comparative abilities

[2] Consistent with this expectation, at pH 2 and below, where the pyromellityl group would be protonated, the CD spectrum of Alm-PM in DOPC vesicles corresponds to that of the self-associated state (data not shown).

to dissipate diffusion potentials created across lipid vesicle membranes. Similar approaches have been used to characterize voltage-dependent channel formation by peptide segments of the inhibitory glycine receptor (Langosch et al., 1991) and by pardaxin and derivatives (Loew et al., 1985; Shai et al., 1990). Small unilamellar vesicles (SUVs) were prepared with a relatively high (200 mM) internal potassium concentration. Upon dilution of the vesicle solution into isotonic medium containing no potassium, an ion gradient is created across the membrane. Addition of the potassium-specific ion carrier valinomycin then results in a diffusion potential across the membrane (*cis* positive, *cis* being the outside and the side of peptide addition). The magnitude of this potential is conveniently monitored using the fluorescent dye safranine O, which associates with the hydrocarbon regions of the lipid vesicles (and thereby undergoes fluorescence enhancement) in a potential-dependent manner (Woolley et al., 1987).

Dissipation of the diffusion potential (via equilibration of ions across the membrane) results in a fluorescence decrease; complete dissipation causes a return to baseline fluorescence, *i.e.*, the fluorescence measured before the addition of valinomycin. If a voltage-independent, nonselective, channel-forming compound were added to the system, a complete dissipation of the potential would be expected (*e.g.*, Loew et al. (1985)). With strongly voltage dependent, nonselective, channel-forming compounds, however, dissipation (*i.e.*, the fluorescence decrease) may be incomplete since the drop in the diffusion potential as ions equilibrate can result in channel closing.[3] The level to which the potential drops may be regarded as a threshold potential analogous to those measured electrically in planar lipid bilayer systems. Below this potential the probability of channel opening is small. The voltage-dependent channel-forming compound may alternatively be added prior to valinomycin; in this case, upon valinomycin addition, the diffusion potential (and fluorescence) increases to a plateau at the threshold potential (G. A. Woolley, unpublished). The threshold potential for alamethicin channel formation depends on peptide concentration (Eisenberg et al., 1973) so that if progressively higher concentrations of alamethicin are added to the energized vesicle system, progressively lower threshold potentials (and fluorescence plateaus) are therefore seen.

Figure 8 (panel A) compares the relative effectiveness of Alm and Alm-PM in dissipating a potassium diffusion potential in vesicles suspended in low ionic strength medium. Addition of 3.3 μM Alm to the system results in an almost complete dissipation of the potential. That is, under these conditions the threshold potential for Alm is near zero. The same concentration of Alm-PM causes a much less significant drop in fluorescence (panel A) indicating a much higher threshold potential. In fact, the concentration of unmodified Alm must be decreased 10-fold in order match this threshold potential (panel B, upper curve).

In view of the observation that calcium ions promoted the self-association of Alm-PM, and because a small addition of calcium would not significantly alter the osmotic balance in the lipid vesicle experiment, we examined the effect of calcium addition on the ability of Alm-PM to dissipate the diffusion potential. The results are presented in Figure 8 (panel B). Whereas the addition of 2 mM calcium (as $CaCl_2$) caused a further significant dissipation of potential in the presence of Alm-PM, it had no effect on the system when only Alm was present. This is consistent with the expectation that added Ca^{2+} should greatly facilitate channel formation by Alm-PM as compared to unmodified Alm because of the greater capacity for ionic regulation of the self-association of the former.

The planar bilayer technique permits direct control of the transmembrane voltage and permits the observation of channel activity at the single-channel level in the absence of other membrane-associated compounds. The observed behavior of Alm-PM in planar bilayers is entirely consistent with the results of the lipid vesicle experiments. The threshold potential for alamethicin channel formation in the planar bilayer system is +30 mV, and for Alm-PM it is +105 mV (Figure 9). Moreover the addition of 50 mM Ca^{2+} (a high concentration since the peptide concentration is more than 2 orders of magnitude lower than in the CD experiments) causes a shift in the threshold potential for Alm-PM to +75 mV; that is, again Alm-PM behaves as a calcium-activated channel-forming peptide. The potency of Alm as a channel former is still greater than that of Alm-PM even when calcium is added. Thus, steric and/or electrostatic differences between the peptides must exist, beyond those which can be overcome by calcium addition.

An examination of Alm-PM single-channel currents before and after $CaCl_2$ addition reveals no major changes (Figure 10; Table 1); the single-channel conductances of equivalent subconductance levels are only slightly changed upon the addition of $CaCl_2$ (Table 1). Although the added ions should contribute to the conductance, both Ca^{2+} and Cl^- have permeabilities significantly lower than that of potassium (P_K/P_{Cl} = 2.7; P_{Ca}/P_{Cl} = 0.3 (Eisenberg et al., 1973)). Neutralization of the charge of the pyromellityl groups by calcium could decrease the local negative surface charge and contribute to lower overall conductances. Since little change occurs in Alm-PM single-channel conductances or lifetimes upon the addition of calcium, the change in the threshold voltage with calcium must be a result of an enhanced probability of channel opening.

Channel formation by Alm-PM can also be facilitated by high concentrations of KCl. Frequent openings of long duration and large conductance are observed over a range of voltages (*e.g.*, +80 mV; Figure 11). Unmodified Alm at the same concentration of KCl is very active but is observed most commonly in the lowest and second-lowest subconductance levels (Figure 11; Table 2). This behavior of unmodified Alm has been reported previously (Hall, 1975; Boheim et al., 1983). In general, monomer–monomer repulsion is expected to be less severe in large channels (high subconductance states) than in small channels (Boheim et al., 1983). Electrostatic repulsion between monomers is expected to be stronger in the Alm-PM case than with unmodified Alm. The observation that higher subconductance levels (large channels) occur commonly with Alm-PM (even in 4 M KCl) is consistent with these expectations.

The lifetimes observed for Alm-PM channels are about an order of magnitude longer than for alamethicin channels in 4 M KCl (Figure 11). In addition, the lifetimes observed for Alm-PM channels in 4 M KCl are considerably longer than those observed in 0.5 M KCl (Figure 10). Variations of subconductance state lifetimes over 1 or 2 orders of magnitude have been reported previously as a function of membrane lipid composition and temperature (Gordon & Haydon, 1976; Sakmann & Boheim, 1979). The variability of alamethicin subconductance state lifetime is at present difficult to relate to any one physical factor.

[3] The alternate explanation for incomplete dissipation—that only a fraction of vesicles are affected—is inconsistent with numerous studies demonstrating the reversibility of alamethicin–vesicle interactions (Rizzo et al., 1987; Archer & Cafiso, 1991; Woolley & Wallace, 1993).

Implications for Physical Models of Channel Formation. The results presented here are consistent with a picture of alamethicin channel formation in which peptides associate with membranes as shown schematically in Figure 13. Self-association is an intrinsic property of the system and is critical for channel formation. We show here how self-association may be modulated by electrostatic repulsion between monomers and have shown previously how self-association can be modulated by temperature (Woolley & Wallace, 1993). These results do not rule out conformational changes that may accompany alamethicin channel gating (*e.g.*, Fox and Richards (1982) and Hall et al. (1983)). It is difficult, however, to account for the observed gating charge of channel formation if one proposes voltage-dependent conformational change as the *sole* factor in channel opening (Sansom, 1993a). An applied voltage may simply alter the relative populations of the species depicted in Figure 12 in favor of transmembrane topologies (*e.g.*, Baumann and Mueller (1974), Schwarz et al. (1986), and Huang and Wu (1991)). Such a process accounts for the observed gating charge of channel formation (Sansom, 1993a) and is consistent with NMR results indicating that alamethicin behaves as a rigid helix (Kelsh et al., 1992). The present data are difficult to reconcile with models for alamethicin channel formation in which flip-flop of the peptide across the membrane is an obligatory step (Boheim et al., 1983). Translocation of the highly charged pyromellityl group would be energetically costly, and changes in the ionic strength of the membrane-bathing solution would be unlikely to facilitate this process.

A further characteristic of Alm-PM channels is that while single-channel events are observed at positive potentials as low as +10 mV (in 4 M KCl), channels are not seen at negative voltages as high as −160 mV, nor do they appear after prolonged measuring times (1–2 h) (not shown). This asymmetry is also difficult to reconcile with flip-flop models of alamethicin channel formation (Boheim et al., 1983). Such flip-flop implies a population of molecules oriented in each direction across the membrane (N-terminus on the *cis* face or vice versa), and although these populations may not be equal, one would nevertheless expect some activity at *cis*-negative potentials if flip-flop was occurring over time. The present results with Alm-PM support the simple view that channels can form through the self-association of alamethicin monomers with all peptide C-termini on one side of the membrane.

Finally, the peptide Alm-PM was not designed to be calcium-activated, and the pyromellityl group is not optimized for Ca^{2+} binding. The present results suggest that by focusing on the process of self-association, it may be possible to design other channel-forming peptides which can be activated more selectively and at much lower concentrations of Ca^{2+} or by other ions.

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