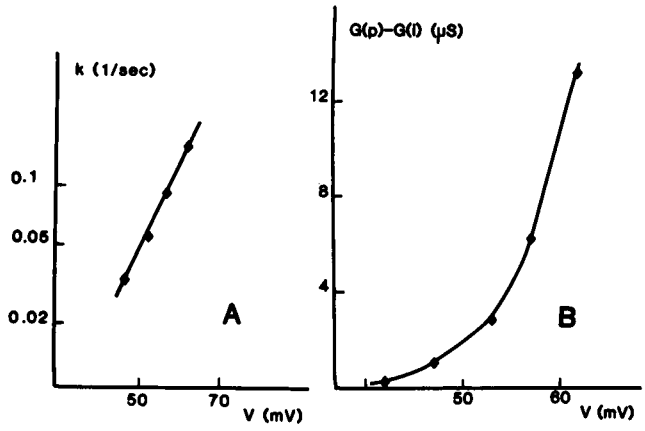

FIGURE 4 Characteristics of the inactivation of the conductance. *A*, rate of inactivation (k, log scale) vs. potential. k was calculated from plots of the log conductance vs. time for the data of the region labeled *i* in Fig. 1 *A*. *B*, voltage dependence of the extent of inactivation as determined from the difference between the steady-state conductance of region *p* (G_p) and the steady-state conductance of region *i* (G_i). Melittin concentration (*cis*-side only): 1.0×10^{-7} M.

inactivation, $(G_p - G_i)$ is also voltage dependent, as illustrated in Fig. 4 *B*.

In summary, the time course of the melittin-induced conductance in bilayers shows two distinct steady-state regions which differ both in the kinetics of activation and in their voltage dependence. One region, *a*, activates rapidly in response to changes in V (time constants of 1–2 ms) and has a gating charge = 1. The other region, *p*, activates more slowly (time constants of 2–10 s) and has a gating charge = 4. The magnitude of the steady-state conductance (at a fixed voltage) in both regions is proportional to the fourth power of the concentration of melittin monomers in the aqueous solution.

Elucidation of the difference in the molecular arrangements responsible for these two regions should shed light on how polymers of L-amino acids can induce ionic conductances in lipid bilayers and, possibly, in biological membranes.

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ION-BOUND FORMS OF THE GRAMICIDIN A TRANSMEMBRANE CHANNEL

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The physical characteristics and conductance properties of gramicidin A, a linear polypeptide antibiotic that forms transmembrane ion channels, have been extensively investigated. The molecule adopts distinctly different conformations in membranes and in organic solvents (1). The membrane-bound form is an amino-terminus-to-amino-terminus helical dimer (2), whereas in organic solvents the molecule appears to form a series of parallel and antiparallel interwined double helices (3), (Fig. 1). The circular dichroism (CD) spectrum of gramicidin in methanol solution is easily distinguishable from that of gramicidin bound to vesicles either by cosolubilization (4) or by heat treatment (5), although both methods of vesicle incorporation produce similar results.

Because gramicidin specifically binds monovalent cations (6, 7), it was of interest to examine the effects of ion binding on the various conformations of the molecule and to determine the relationship of the ion-bound forms to the channel structure. Binding of Cs^+ to the membrane-bound form results in no change in the peptide backbone conformation (4), whereas binding to the methanol solution structures produces not only a change in pitch of the helix, but also a net change in handedness of at least the predominant form of double-helix dimer (8, 1). None of the conformations in methanol (either with or without Cs^+) resembles the membrane-bound form. Even when gramicidin is loaded with Cs^+ in a methanol solution prior to binding to vesicles, the spectrum of the incorporated

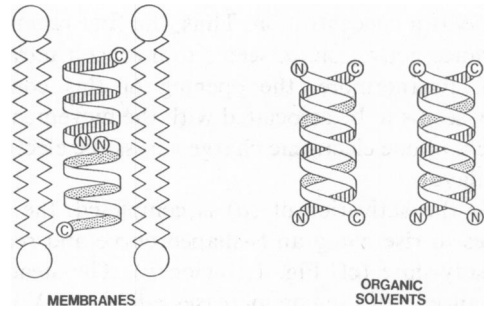

FIGURE 1 Schematic diagram of the conformations of gramicidin A in phospholipid bilayers and in organic solvents.

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form corresponds to molecules largely in the "membrane" conformation rather than in the "Cs^+-solution" conformation. Under very high salt conditions, initially some of the Cs^+ solution form is seen for the vesicle-bound, but not incorporated gramicidin; however, this is converted to the membrane form upon incorporation into the bilayer, suggesting that the stable ion-bound form of the integral membrane channel is very different from either the ion-free or the ion-bound forms in solution in the absence of lipids.

METHODS

Stock solutions of gramicidin A (3 mg/ml) (ICN Nutritional Biochemicals, Cleveland, OH) either with or without CsCl (100 mM in spectrograde methanol, Fisher Scientific Co., Pittsburgh, PA) were prepared; these were diluted 1:10 with either 100 mM CsCl in methanol or with vesicle solutions (see below). At these concentrations, the gramicidin molecules are saturated with cesium (8); no changes are seen in the gramicidin solution spectrum with time or after heating to 60°C.

Small sonicated dimyristoyl phosphatidylcholine vesicles (~500 Å by negative-stain electron microscopy) were prepared as previously described (4). The light-scattering and absorption flattening properties of these small particles is minimal (4) and will not significantly distort the resulting spectra. Vesicles were diluted 1:1 either with water or 4M or 20 mM aqueous solutions of CsCl. Gramicidin-containing vesicles were prepared by addition of 10λ of the gramicidin stock solution in methanol (either with or without CsCl), to 90 μl of the vesicle suspension. The samples were incubated for 20 min at 23°C. Some of the samples were further heated for 3 h at 60°C.

CD spectra were obtained using a Cary 60 spectropolarimeter with a model 6001 CD attachment and a variable position detector. Measurements were made at 23°C using a 0.5 mm pathlength cell; the photomultiplier was located directly adjacent to the sample cell to reduce scattering effects. At least three scans of two independent samples were done for each specimen. Gramicidin concentrations were determined from the OD_{282} (ϵ = 22,000).

RESULTS AND CONCLUSIONS

Circular dichroism spectroscopy is useful for characterizing the secondary structure of polypeptides. The net spectra obtained for gramicidin in methanol solution and in dimyristoyl phosphatidylcholine vesicles are distinctly different (Fig. 2). The spectrum in methanol has been shown to arise from a mixture of four slowly interconverting conformers, which has been described as a family of intertwined double helices (3) whose chains run either parallel or anti-parallel and may be of different helical senses. Both left- and right-handed helices are possible because of the alternating l- and d- nature of the amino acids which comprise this molecule. Similar spectra are obtained in a wide range of organic solvents (4). However, the spectrum of gramicidin in lipid vesicles is very different from any of these (Fig. 2), although similar results were obtained for vesicles produced by either cosolubilization (4) or heat treatment (5). The vesicle spectra cannot be produced by any linear combination of the spectra of the conformers in organic solvents, supporting the notion that different conformations are present in solutions and in vesicles.

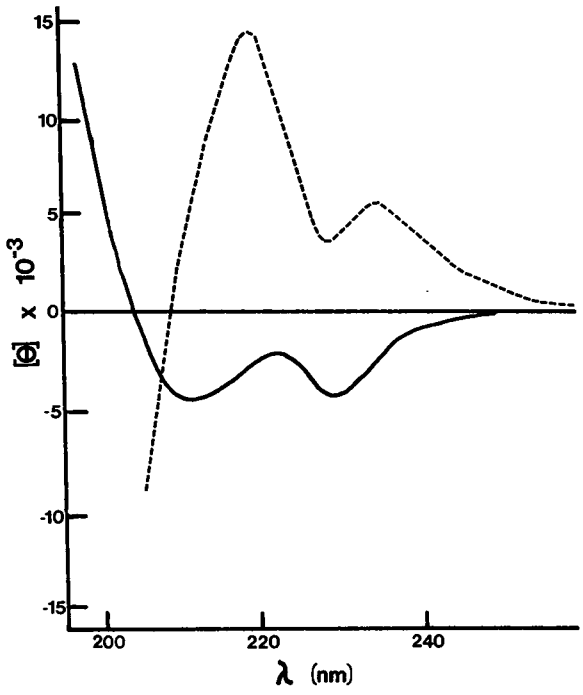

FIGURE 2 Circular dichroism spectra of gramicidin A in methanol solution (—) and in dimyristoyl phosphatidylcholine vesicles (---).

Because gramicidin acts as an ion channel, the interactions of the different conformations with monovalent cations may provide information on its mechanism of action. When the conformation in methanol binds Cs^+, its CD spectrum changes dramatically (Fig. 3). The altered sign and shape of the curve suggests not only a change in folding motif, but also a change in handedness (8). Different ion sizes produce spectra of different maximum ellipticities, which can be correlated with altered pitches of the helices (8). In contrast, when Cs^+ binds to gramicidin that has been incorporated into vesicles, there is no overall change in the backbone structure of the polypeptide (compare Figs. 2 and 3), even at cation concentrations where the channel sites are fully saturated (7). Therefore, the forms of gramicidin in methanol and in vesicles appear to have substantially different modes of interaction with ions.

It was also of interest to see if conditions could be found under which the "Cs^+-solution" form of gramicidin could be incorporated into membranes. This was examined by preincubation of gramicidin in methanol solutions containing saturating CsCl concentrations. The gramicidin solution containing the Cs^+-solution form of the molecule was then added to vesicle suspensions. Under these conditions, the gramicidin is bound to the vesicles (5); it is then incorporated into the bilayer by heat treatment (5). This method is analogous to one procedure used to incorporate gramicidin into black lipid films used for conductance measurements (6). (Previous cosolubilizations studies were done under conditions similar to those used in other conductance studies [9]). The spectrum obtained immediately following addition to vesicles (surface binding condi-

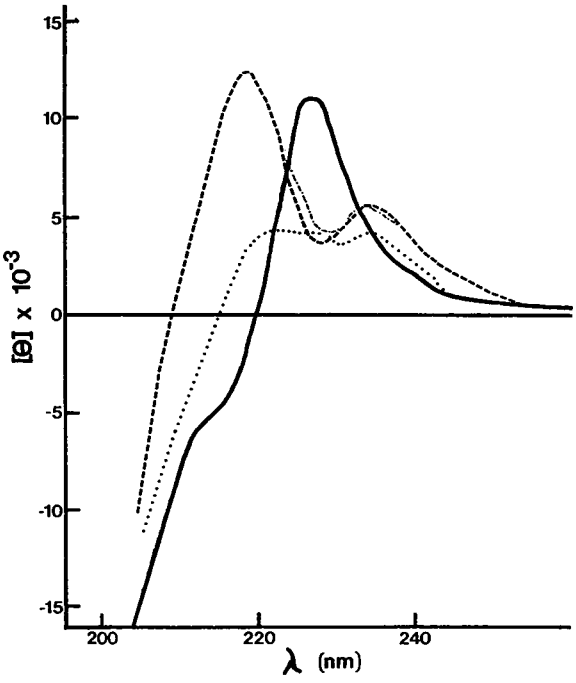

FIGURE 3 Circular dichroism spectra of cesium-bound forms of gramicidin A: gramicidin and CsCl in methanol solution (100 mM CsCl) (—), CsCl (2M) added to preformed DMPC-gramicidin vesicles (---), and gramicidin preloaded with cesium in methanol solution and then added to DMPC vesicles in H_2O, prior to heating (bound) (· · ·), and after heating for 3 h at 60°C (incorporated) (-.-.-.).

tions) exhibited features associated with both Cs^+ solution and vesicle forms (Fig. 3), and corresponded roughly to a linear sum (1:2) of those two spectra. When the sample was heated (which results in bound molecules becoming incorporated into the bilayer as active channels), the spectrum converted entirely to that of the vesicle form (Fig. 3), suggesting that Cs^+-solution conformation is not the form of the channel in membranes. However, since CsCl is more soluble in water than in the gramicidin/vesicle complex, it may partition into the aqueous phase, leaving the gramicidin in an uncomplexed form, so no ion effect would be seen. Therefore, the studies were repeated with vesicles in 100 mM, 200 mM, and 2 M aqueous CsCl solutions. At 2 M the channel is fully saturated with Cs (7). In these three cases, the proportion of the Cs^+-solution form in the initial (bound) spectrum is increased (up to 1.4:1 in the case of 2M Cs^+); however, upon incorporation, all also revert to the vesicle-form spectrum.

These results suggest that the ion-bound form of the active membrane channel is nearly identical to the ion-free form of the membrane channel, and is totally dissimilar from the ion-bound form in methanol solution. Although the Cs^+-solution form can apparently be associated with the membrane surface, after incorporation into the bilayer the molecule is transformed to the vesicle conformation. Thus, the Cs^+-solution form (presumably a double helix) does not appear to exist as an membrane-active structure. This further suggests that crystals formed from Cs^+-methanol solutions, presently under study in a number of laboratories,[1] (10) are unlikely to represent the channel form, although crystals formed in the presence of lipid (1) do appear to adopt a conformation similar to that in membranes.

In summary, it appears that no major structural differences exist between the ion-bound and ion-free forms of gramicidin in membranes, and that these conformations are unrelated to either the ion-bound or ion-free conformations of the molecule in organic solvents.

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