Gramicidin single-channel properties show no solvent-history dependence

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ABSTRACT The structure of membrane-associated gramicidins can depend on the solvent in which they were dissolved prior to membrane incorporation (LoGrasso, P. V., F. Moll, and T. A. Cross. 1988. *Biophys. J.* 54:259–267; Killian, J. A., K. U. Prasad, D. Hains, and D. W. Urry. 1988. *Biochemistry.* 27:4848–4855). The peptide's solvent history might thus affect the functional characteristics of gramicidin channels (op. cit.). We tested this proposal by examining the properties (conductance, conductance dispersity, and average duration) of channels formed by [Val^1]gramicidin A that had been dissolved in eight different solvents. The peptide was incorporated into lipid bilayers either by addition to the aqueous phase (and subsequent adsorption to the membrane) or by cosolubilization with the membrane-forming phospholipid. When the peptide was cosolubilized with the phospholipid, the channel properties did not vary with the solvent used. When the peptide was dissolved in chloroform, benzene, or trifluoroethanol and added through the aqueous phase, the channel properties differed from those found when gramidicin was dissolved in methanol, ethanol, dioxane, dimethylsulfoxide, or ethylacetate. The changes observed with the former three solvents were reproduced by adding them to the aqueous phase, and are therefore due to the ability of these solvents to partition into the membrane and alter the channels' behavior.

INTRODUCTION

The primary structures of many proteins are now readily available, but protein secondary structural determinations remain difficult, particularly for integral membrane proteins. Moreover, even when spectroscopic techniques can provide information about protein secondary structures, it may not be clear how this information should be related to a protein's function. Here we illustrate this problem in using the channel-forming peptide [Val^1]gramicidin A. We find that experimental maneuvers that produce variations in the membrane structure of gramicidin (at peptide/lipid ratios ~1:15), as measured by nuclear magnetic resonance (NMR) and circular dichroism (CD) spectroscopy (LoGrasso et al., 1988; Killian et al., 1988*b*), do not produce comparable variations in gramicidin channel function (at peptide/lipid ratios $\sim 10^{-7}$).

[Val^1]Gramicidin A is a linear pentadecapeptide with the sequence: HCO-L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-D-Leu-L-Trp-NHCH$_2$CH$_2$OH (Sarges and Witkop, 1965). The structure and function of gramicidin channels have been studied extensively (for recent reviews see Wallace, 1986; Andersen et al., 1988). It is generally agreed that the channels predominantly are made up of two $\beta^{6.3}$-helical monomers associated in a formyl-NH$_2$-terminal-to-formyl-NH$_2$-terminal dimer (as first proposed by Urry [1971]), which forms a cation-selective channel that can span lipid bilayers. CD spectra of gramicidin in phospholipid vesicles have a characteristic shape that is believed to result from peptides that have folded into $\beta^{6.3}$-helices (Masotti et al., 1980; Wallace et al., 1981). This CD pattern is known as the "channel" spectrum, but this does not imply that it is caused solely by conducting dimers, because gramicidins that have been modified so as to form dimers poorly, if at all, still can give rise to the "channel" spectrum (Killian et al., 1988*b*).

In organic solvents the gramicidins exist in several additional forms that are in (slow) equilibrium with one another (Veatch and Blout, 1974). These "solvent structures" vary with the solvent, gramicidin concentration, and temperature (Veatch and Blout, 1974; Urry et al., 1975; Sychev et al., 1980; Wallace et al., 1981), and the interactions of gramicidin with lipid bilayers vary in concert with the changes in solvent structure. When dissolved in trifluoroethanol or dimethylsulfoxide, for example, gramicidin is monomeric (Glickson et al., 1972; Hawkes et al., 1987). When incorporated into membranes from these solvents, it seems to go directly into the channel form (Masotti et al., 1980; Tournois et al., 1987). By contrast, when gramicidin is incorporated from solvents such as chloroform or ethanol, in which it tends to form various intertwined dimers, the "channel" structure

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0006-3495/90/03/515/09 $2.00

is slow to appear (Masotti et al., 1980; Killian et al., 1988*a* and *b*; LoGrasso et al., 1988).

The question remains whether the structure (and properties) of the membrane-spanning channels vary as a function of the peptide's solvent history. To examine this question we studied the properties of [Val^1]gramicidin channels in planar lipid bilayers. [Val^1]Gramicidin A was incorporated into membranes from eight different organic solvents and the single-channel properties were evaluated.

Some of these results have appeared in preliminary form (Sawyer et al., 1989*a*).

MATERIALS AND METHODS

Diphytanoylphosphatidylcholine (DphPC) was from Avanti Polar Lipids, Inc. (Birmingham, AL), and was further cleaned by ion-exchange chromatography (Andersen, 1983). *n*-Decane was from Wiley Organics (Columbus, OH), and was used without further purification. Gramicidin D, a mixture of the naturally occurring gramicidins A, B, and C, was from Sigma Chemical Co. (St. Louis, MO). [Val^1]Gramicidin A was purified from the commercial mixture as described previously (Koeppe and Weiss, 1981). [F_3Val^1]Gramicidin A was prepared from gramicidin D via the intermediates Des(formyl-valyl-gramicidin and *N*-formyl-4,4,4-trifluorovaline, as has been described previously (Weiss and Koeppe, 1985; Russell et al., 1986). Ethanol (absolute) was from United States Industrial Chemicals (Tuscola, IL); 2,2,2-trifluoroethanol (purissimum) was from Fluka AG (Buchs, Switzerland); benzene (>99.9% pure) was from Fisher Scientific Co. (Fair Lawn, NJ); methanol (>99.9% pure), chloroform (>99.9% pure), ethylacetate (>99.5% pure), dimethylsulfoxide (>99.5% pure), and dioxane (>99.9% pure) were from Burdick & Jackson Laboratories Inc. (Muskegon, MI). NaCl was suprapure grade from E. Merck (Darmstadt, FRG) and water was deionized milli-Q water (Millipore Corp., Bedford, MA). The NaCl was roasted at 500°C for 24 h and stored over NaOH in evacuated desiccators.

The general experimental procedures were as described in Andersen (1983). The experiments were done with symmetrical unbuffered solutions of 1.0 M NaCl. The temperature was maintained at 25 ± 2°C by water circulating in an external jacket. Planar lipid bilayers were formed across a hole (diameter ~1.6 mm) in a Teflon partition separating two identical Teflon chambers. [Val^1]Gramicidin A was incorporated into the membranes using two different techniques: either by adding the peptide, dissolved in the chosen solvent, to the electrolyte surrounding the membrane and allowing it to adsorb and incorporate into the bilayer, or by cosolubilizing peptide and lipid together in the appropriate solvent before making the membrane-forming solution.

In the first series of experiments, the membrane-forming solution was diphytanoylphosphatidylcholine (DPhPC) in *n*-decane (2.5% wt/vol). [Val^1]Gramicidin A was dissolved in methanol, diluted 1:100 in the chosen solvent (to a final concentration ~10 nM) and equilibrated for at least 1 h. Aliquots of these dilutions were then added in equal amounts to both aqueous phases during vigorous stirring. The actual amount of the peptide stock that was added varied from 10 μl (for ethanol, methanol, ethylacetate, dimethysolfoxide, trifluoroethanol, and dioxane) to 15 μl (for benzene and chloroform).[1] The stirring was continued for >1 min before the measurements began. The nominal peptide concentration in the electrolyte solution was ~10 pM.

In the second series of experiments, [Val^1]gramicidin A was diluted 1:100 in ethanol and added to DPhPC stored in chloroform to achieve a peptide/lipid molar ratio of 10^{-7}. This solvent mixture was evaporated under N_2, and the peptide-lipid mixture was redissolved in the chosen solvent (to a peptide concentration ~2 nM) and equilibrated for at least 1 h. The solvent was again evaporated, the mixture was resuspended in *n*-decane (2.5% wt/vol), and planar bilayers were formed. All single-channel recordings were completed within 2 h of the solvent evaporation.

In experiments where the effect of added solvent was investigated, a basic channel activity was established by first adding gramicidin A dissolved in ethanol to both aqueous phases. After control readings were obtained, 10-μl aliquots of the appropriate (gramicidin-free) solvent were added to both aqueous phases during vigorous stirring, to reach nominal concentrations comparable with those in the first set of experiments. These experiments were carried out on [Val^1]gramicidin A, as well as [F_3Val^1]gramicidin A.

Single-channel measurements were done at 100 mV applied potential (200 mV for [F_3Val^1]gramicidin A channels) using the bilayer punch (Andersen, 1983). Single-channel current transitions were detected and their amplitude was determined as the difference between the average current levels in 40-ms-long records collected just before and just after each current transition (Andersen, 1983). No attempt was made to match channel appearance events with channel disappearance events. To ensure that low-amplitude current transitions would be incorporated into the sample, the discrimination level that triggers transition detection was set sufficiently low that baseline current noise would trigger a significant number of "transitions." Each experiment consisted of two to six consecutive measurements based on 500 current transitions, where typically 100–300 were resolved and displayed in current transition histograms. Records were eliminated from the final data set if there was poor small membrane stability, if the channel activity was too high, or if there was evidence for increased series resistance in the pipette.

Determinations of current transition amplitudes and "mini" channel frequencies were always done based on current transition histograms. Gramicidin channels are not completely homogeneous (Busath and Szabo, 1981). In our laboratory, typically 95% of channels formed by [Val^1]gramicidin A have conductances that fall within a narrow Gaussian distribution in a current transition histogram (Busath et al., 1987; Sawyer et al., 1989*b*). The remaining 5% of resolved current transitions have amplitudes below the main distribution, and are referred to as "mini-channels." We believe they represent subtle conformational changes in the conducting structure (Sawyer et al., 1989*b*). The percentage of events that are within the main distribution, therefore, is a measure of channel homogeneity. The number of normal and mini-current transitions were determined for each record, based on inspection of the current transition amplitude histograms. The distinction between normal and mini-current transitions was made using the criteria outlined in Busath et al. (1987). The mini-frequency was calculated as the number of events more than three standard deviations from the average current value for the main peak, divided by the total number of events in the current transition histogram, multiplied by 100.

(During the time these experiments were done, a major construction project was underway in the immediate vicinity of the laboratory. This produced substantial building vibrations and temperature variations,

[1]When [Val^1]gramicidin A was dissolved into benzene or chloroform, single-channel measurements had to be performed within several hours of the dilution to detect any channels. After that time, there was no detectable peptide in these dilutions as measured by fluorescence absorption (kindly done by Dr. S. Scarlata), whereas there was plenty of gramicidin in an ethanolic solution. When dissolved in very nonpolar solvents, it thus appears that gramicidin adsorbs to the walls of the glass vial in which it is stored.

which are manifested in variable quality of the current records and increased standard deviations of the main peaks of some current transition histograms. We do not believe these problems alter the interpretation of our findings).

Channel durations were determined by matching channel appearances with disappearances and measuring the interval between these. Only events whose amplitude fit within the desired (main) distribution in the amplitude histogram for that experiment were used in the analysis. Where channels occurred singly this was straightforward. When a new channel appeared while one (or more) channels were conducting, channel appearances and disappearances that fell within the limits of the amplitude distribution were matched using a randomized assignment. This is valid as long as all appearances (and disappearances) of the same amplitude represent the same type of molecular event. Channel durations were plotted as normalized survivor plots (e.g., Andersen and Muller, 1982). To estimate the average duration, τ, of the distributions, single exponential decays, $N(t)/N(0) = \exp(-t/\tau)$, were fitted to the results using a maximum likelihood estimator (Hall and Sellinger, 1981), where $N(t)$ denotes the number of channels with a duration $\geq t$, and $N(0)$ is the estimated total number of channels in the population. $N(0)$ differs from the number of events actually observed, because the on-line channel detection algorithm will not define unambiguously events with durations <40 ms; such events may thus be excluded in the subsequent analysis.

RESULTS

Fig. 1 shows single-channel current traces and current transition histograms for channels formed when [Val^1]-gramicidin A is added to the electrolyte from eight different solvents. In all cases, >90% of the channels in the amplitude histogram fit within a Gaussian distribution, with channel durations that are well fit by a single exponential decay for more than three time constants (see also Fig. 3). The channels outside of the main amplitude distribution are "mini" channels which in all cases are similar to those described in detail previously (Sawyer et al., 1989*b*). When the peptide is added from ethanol,

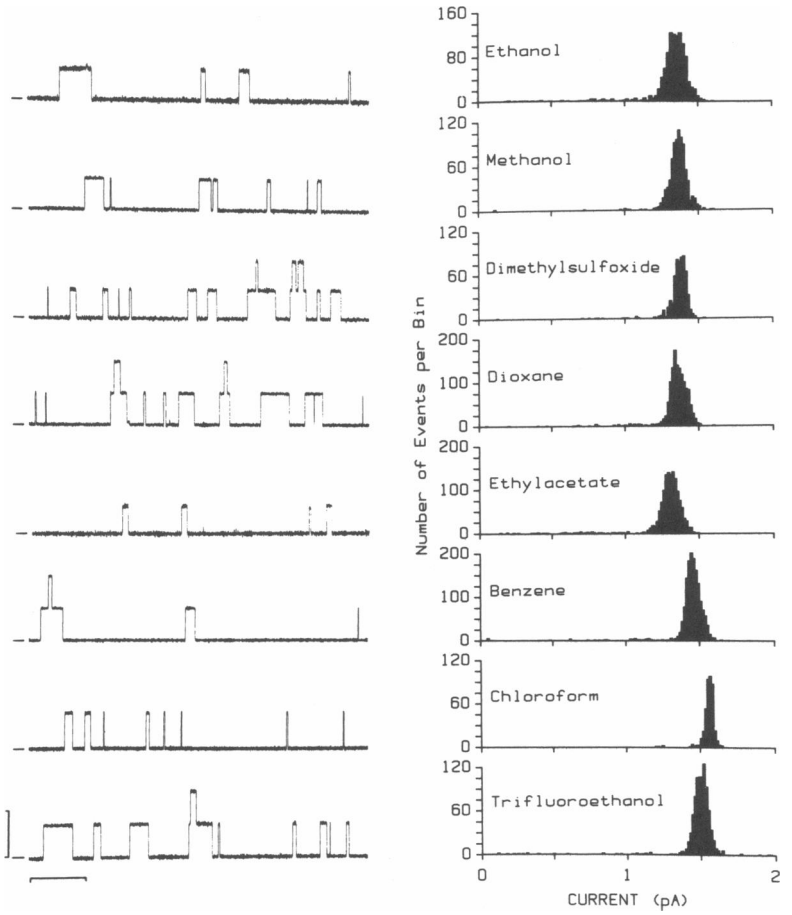

FIGURE 1 Single-channel behavior induced by [Val^1]gramicidin A dissolved in eight different solvents. The peptide was dissolved in the chosen solvent, equilibrated for at least 1 h, and added to the electrolyte solution surrounding the bilayer. (*A*) Single-channel current traces. (*B*) The corresponding current transition amplitude histograms. Statistics for the experiments are summarized in Table 1. Note the increase in channel amplitude when the peptide was dissolved in chloroform, benzene, and trifluoroethanol. Vertical calibration bar denotes 2 pA; horizontal bar, 5 s. Current traces were filtered at 40–70 Hz.

methanol, ethylacetate, dimethylsulfoxide, or dioxane, the channels are indistinguishable. There are no obvious differences in channel behavior among the current traces, nor are there statistically significant differences in the current transition distributions or the average channel durations (Table 1).

When [Val^1]gramicidin A is added from benzene, chloroform, or trifluoroethanol the channel properties vary with the solvent used. For all three solvents, the single-channel transition amplitudes are significantly larger than when using the five solvents listed above. When [Val^1]gramicidin is dissolved in benzene or chloroform, the channels also have shorter average durations (Table 1).

In these experiments some solvent is added along with the peptide. The solvent-dependent variations in channel properties could thus result because the solvents alter the channel behavior only indirectly, by altering the properties of the host bilayer. To examine this question, [Val^1]-gramicidin A was cosolubilized with DPhPC and equilibrated in the chosen solvent before the solvent was evaporated and *n*-decane was added. In these experiments the channels behave identically regardless of the solvent used (Fig. 2). ([Val^1]Gramicidin A was not cosolubilized with lipid in dimethylsulfoxide because we could not completely evaporate this solvent). As was the case in Fig. 1, there were some experiment-to-experiment variations. But they are no larger than the usual day-to-day variations (cf. Busath et al., 1987; Sawyer et al., 1989*b*).

TABLE 1 Single-channel parameters for gramicidin A that has been dissolved in different solvents

Solvent	Mode*	Current	N_t‡	$\%_h$§	τ‖	$N(0)$‖	N_c‖
		mean pA ± SD			*ms*		
Ethanol	A	1.36 ± 0.07	1087	94	750	670	615
	B	1.34 ± 0.06	1322	95	840	480	480
Methanol	A	1.36 ± 0.05	845	98	870	435	403
	B	1.26 ± 0.08	1463	95	830	770	705
DMSO¶	A	1.30 ± 0.06	1801	96	810	330	314
	B	—	—	—	—	—	—
Dioxane	A	1.36 ± 0.05	1335	94	960	470	467
	B	1.36 ± 0.05	882	94	950	600	602
Ethylacetate	A	1.30 ± 0.06	1378	95	900	720	686
	B	1.27 ± 0.05	1622	92	830	830	790
Benzene	A	1.45 ± 0.05	1563	94	630	770	696
	B	1.33 ± 0.07	1411	93	790	680	648
Chloroform	A	1.55 ± 0.03	423	98	280	500	437
	B	1.30 ± 0.06	1319	97	870	580	536
TFE**	A	1.50 ± 0.05	914	97	950	420	406
	B	1.37 ± 0.06	1545	96	900	860	770

*Mode of incorporation of gramicidin into membranes: (*A*) addition of peptide and solvent to the electrolyte solution (corresponding to results shown in Fig. 1). (*B*) Cosolubilization of gramicidin and lipid in solvent, where solvent was evaporated before membrane formation (corresponding to results shown in Fig. 2 [see Methods]).

‡Total number of current transitions shown in the histogram of Fig. 1 or 2.

§Percentage of the total number of current transitions that fall within the normal distribution described by "mean ± SD."

‖Mean channel duration (τ) and the initial number of channels open N(0) are computed from the equation $N(t) = N(0)\exp(-t/\tau)$, used to describe the set of N_c channels of measured duration with the maximum likelihood estimator (see Methods).

¶Gramicidin A was not cosolubilized with DPhPC in dimethylsulfoxide (DMSO) as this solvent could not be completely evaporated.

**Trifluoroethanol.

A comparison of the results in Figs. 1 and 2 suggests that the effects of benzene, chloroform, and trifluoroethanol seen in Fig. 1 result from an indirect (membrane-mediated) effect of the added solvents. This possibility was tested further by adding these solvents directly to the electrolyte solutions surrounding a bilayer in which [Val^1]gramicidin A was already incorporated from an ethylacetate or ethanol stock solution. Addition of 40 μl of ethanol to the electrolyte had no effect on channels formed by [Val^1]gramicidin A that was added to the membrane from an ethylacetate solution. In all these other experiments the peptide was added from ethanol. To minimize the impact of day-to-day variations, the measurements after solvent addition were compared to control measurements from the same day just before solvent addition. In each case, adding the solvent at volumes comparable with those used for the experiments in Fig. 1 produced effects on the channel behavior that were similar to those obtained when the peptide was dissolved in that solvent (Fig. 3; Table 2). For trifluoroethanol, the channel amplitude increased, while the duration remained unchanged. For benzene and chloroform, the channel amplitude increased, while the duration decreased.

In an attempt to better understand the basis for these solvent effects, we also examined the behavior of channels formed by [F_3Val^1]gramicidin A. These channels have a substantially smaller conductance than [Val^1]gramicidin A channels (Russell et al., 1986), which should make their conductance more sensitive to changes in the membrane environment.

Similar to the results obtained with [Val^1]gramicidin A, chloroform and benzene decreased the duration of [F_3Val^1]gramicidin A channels, whereas trifluoroethanol had no effect on channel duration (Table 2). The most dramatic differential effect was on the conductance changes induced by chloroform, which causes a 30% increase in the conductance of [F_3Val^1]gramicidin A channels, but only a 10% increase for [Val^1]gramicidin A channels (Table 2). The conductance increases induced by benzene and trifluoroethanol were less and not clearly different from those found with [Val^1]gramicidin A channels (Table 2).

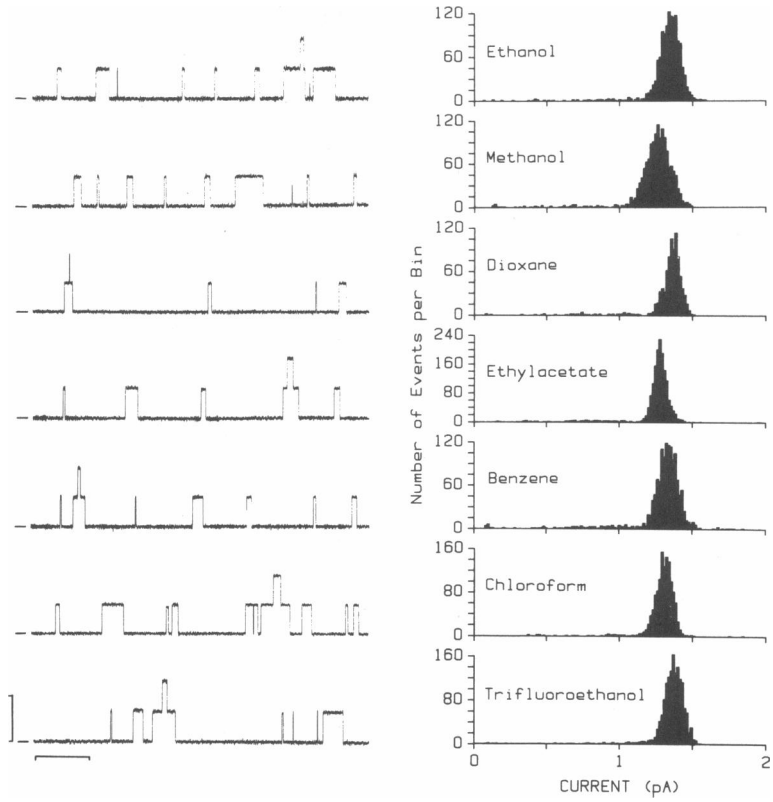

FIGURE 2 Single-channel behavior induced by [Val¹]gramicidin A when codissolved with DPhPC in seven different solvents. The peptide A was codissolved in the chosen solvent along with the lipid at a peptide/lipid ratio $\sim 10^{-7}$. (*A*) Single-channel current traces. (*B*) Corresponding current transition amplitude histograms. Statistics for the experiments are summarized in Table 1. Vertical calibration bar denotes 2 pA; horizontal bar, 5 s. Current traces were filtered at 40–70 Hz.

DISCUSSION

The main result in this article is that the behavior of channels formed by [Val¹]gramicidin A in planar lipid bilayers does not depend on the peptide's solvent history. This result is in apparent contrast with that obtained in spectroscopic studies, where the peptide structure in lipid vesicles depends on the solvent in which it was dissolved before membrane incorporation (Killian et al., 1988*b*; LoGrasso et al., 1988). A likely cause of this difference[2] is the much lower peptide/lipid ratios that are used in single-channel studies ($\sim 10^{-7}$) relative to those used in spectroscopic studies (0.01–1). It has, in fact, been found that the solvent dependence of the channel structure becomes less marked as the peptide/lipid ratio is decreased from 0.1 to 0.02 (Killian et al., 1988*b*). A comparison of the results of Wallace et al. (1981) led likewise to the conclusion that decreasing the peptide/lipid ratio favors the channel structure over any nonchannel structures (LoGrasso et al., 1988). Hence at the extremely low peptide concentrations used in functional studies, only one structure might exist, i.e., the "channel structure" (nonconducting monomers or conducting dimers of $\beta^{6.3}$-helices), but see below.

It should also be noted that single-channel studies, by definition, provide direct information only about those peptides that form conducting channels. In spectroscopic studies, however, all conformational species will contribute to the spectra, with those present in highest concentration being dominant regardless of their functional activity. Nonconducting gramicidin conformers are "invisible" in our experiments, which implies that the dominant

[2]Decane, or other hydrocarbon solvents, present in the membranes used for functional studies of gramicidin channels could possibly disrupt any nonchannel gramicidin structures. This is unlikely, however, because current transition histograms of gramicidin channels (added from ethanolic solution) in lipid bilayers with no hydrocarbon solvent are indistinguishable from those shown here (Sawyer, D. B., and O. S. Andersen, manuscript in preparation).

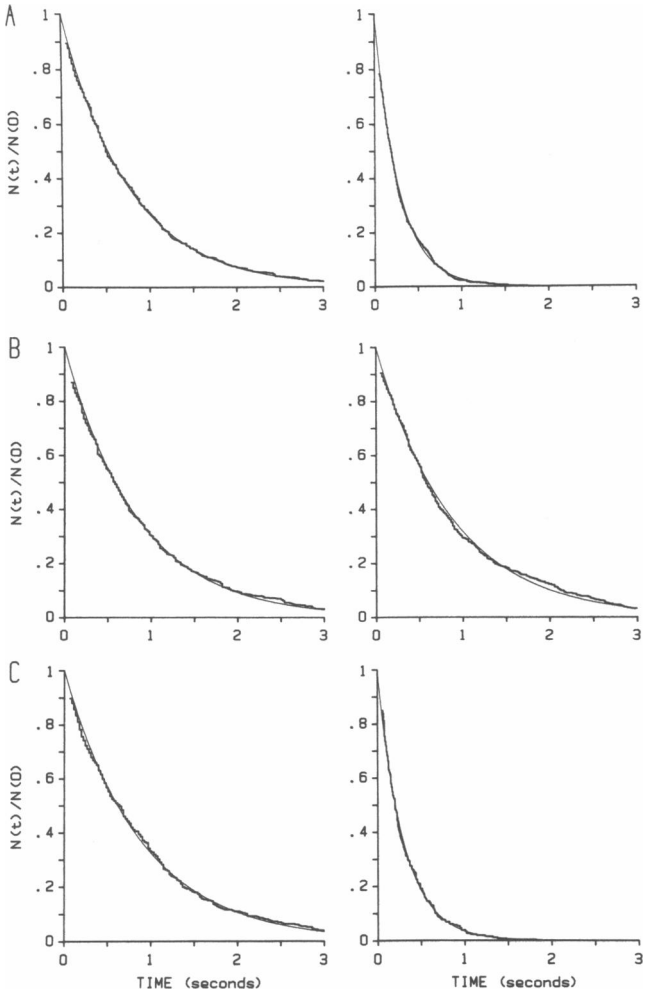

FIGURE 3 Normalized survivor plots of gramicidin A channel durations in DPhPC/decane membranes. (*A*) Gramicidin A dissolved in ethanol (*left*) or chloroform (*right*). All channels are from the main peak of the corresponding amplitude histograms in Fig. 1, with statistics presented in Table 1 (Mode A). (*B*) Gramicidin A cosolubilized with DPhPC in ethanol (*left*) or chloroform (*right*). All channels are from the main peak of corresponding amplitude histograms in Fig. 2, with statistics presented in Table 1 (Mode B). (*C*) Gramicidin A dissolved in ethylacetate with 129 mM ethanol subsequently added to the aqueous solution (*left*) and gramicidin A dissolved in ethanol with 25 mM chloroform subsequently added to the aqueous solution (*right*). All channels are from the corresponding experiments in Table 2. Curves denote the fits of single exponential decays to the results.

TABLE 2 Effect of added solvents on [Val^1]gramicidin A and [F_3Val^1]gramicidin A

Solvent	Current	N_t*	%$_h$‡	τ§	$N(0)$§	N_c§
	mean pA ± SD			*ms*		
[Val^1]Gramicidin A:						
Control	1.39 ± 0.04	274	89	760	170	162
+ ethanol	1.36 ± 0.04	645	98	884	350	320
Control	1.24 ± 0.04	1237	97	760	670	615
+ benzene	1.34 ± 0.03	327	93	540	160	138
Control	1.36 ± 0.07	1087	94	750	620	527
+ chloroform	1.58 ± 0.09	453	90	290	260	227
Control	1.36 ± 0.05	845	99	870	435	403
+ TFE	1.56 ± 0.05	733	97	870	450	429
[F_3Val^1]Gramicidin A:‖						
Control	0.84 ± 0.08	293	85	290	130	116
+ benzene	0.96 ± 0.10	411	87	260	160	155
Control¶	0.86 ± 0.06	897	90	370	440	418
+ chloroform	1.16 ± 0.06	389	86	110	240	184
Control¶	0.86 ± 0.06	897	90	370	440	418
+ TFE	0.94 ± 0.04	463	86	420	270	238

*Total number of current transitions recorded.
‡Percentage of the total number of current transitions that fall within the normal distribution described by "mean ± SD."
§Mean channel duration (τ) and the initial number of channels open $N(0)$ are computed from the equation $N(t) = N(0)\exp(-t/\tau)$, used to describe the set of N_c channels of measured duration with the maximum likelihood estimator (see Methods).
‖Recordings of channels formed by [F_3Val^1]gramicidin A were done at 200 mV applied potential.
¶These control results are pooled from two experiments done on the same day.

species detected in spectroscopic studies need not necessarily be an active (i.e., conducting) structure.

The molecular basis for the solvent dependence of the membrane-incorporated gramicidin structure is most likely related to the tendency of linear gramicidins to form intertwined dimers in nonpolar solvents, and the slow interconversion among these dimers (Veatch and Blout, 1974). When gramicidin is dissolved in solvents that favor the monomeric state (e.g., triflluoroethanol), the membrane-incorporated peptides fold immediately into β-helices, and the CD spectrum of the membrane-incorporated peptide is of the "channel" type (Killian et al., 1988*b*; LoGrasso et al., 1988). When gramicidin is dissolved in solvents that favor the dimeric state (e.g., ethanol), the membrane-incorporated peptides cannot fold into β-helices until the intertwined dimers have become unwound. This is a slow process, and the presence of the intertwined dimers has been demonstrated directly by size-exclusion high-performance liquid chromatography (Bañó et al., 1989). One would thus expect that the CD spectrum of the membrane-incorporated peptide should resemble that of the dissolved peptide, at least for short times after incorporation (but to our knowledge that has not been demonstrated directly). With time (Bañó et al., 1989), or after heating the samples (Killian et al., 1988*b*; LoGrasso et al., 1988; Bañó et al., 1989), the CD pattern of the membrane-incorporated peptide changes slowly towards the "channel" spectrum. These changes in the CD pattern are associated with the almost complete

disappearance of the intertwined dimers (Bañó et al., 1989). The solvent history dependence of the structure of membrane-incorporated gramicidins thus appears to be the result of a kinetic "trap," which occurs because of an extremely slow rate of unwinding and dissociation of the intertwined dimers.[3] The equilibrium structure of membrane-incorporated gramicidin seems to be the "channel" structure, i.e., the $\beta^{6.3}$-helix in monomer-to-dimer equilibrium. Not surprisingly, therefore, gramicidin channels exhibit no solvent history dependence. It should also be noted that the gramicidin concentrations in the stock solutions used here were so low, ~10 nM, that the monomer-dimer equilibrium would be shifted in favor of the monomers before adding the molecules to the membrane.

The question remains whether there may be other (non-$\beta^{6.3}$-helical) membrane-incorporated monomeric structures (cf. Cifu, A., R. E. Koeppe, and O. S. Andersen, manuscript in preparation). There is little direct experimental information pertaining to this question, which is of considerable importance because the membrane-incorporated gramicidins occur almost exclusively as monomers at the peptide/lipid ratios used in single-channel and most other functional studies.[4]

In any case, these results highlight the difficulties inherent in comparing the results from spectroscopic and single-channel studies. Moreover, if the average structure itself varies with concentration, such correlations become even more difficult, because the low channel densities that are necessary for accurate functional assays usually will be inadequate for structural studies, where high peptide concentrations are needed to produce detectable signals.

[3]The rate of unwinding and dissociation of membrane-incorporated intertwined dimers is less than the interconversion rate for gramicidins in organic solvents (cf. Bañó et al. [1989] and Veatch and Blout [1974]. In organic solvents, however, the interconversion rate decreases as the solvent polarity decreases (Veatch and Blout, 1974). The slow disappearance of the membrane-incorporated intertwined dimers could thus result because they penetrate quite deeply into the hydrocarbon core of the membrane, consistent with the calorimetric results of LoGrasso et al. (1988).

[4]The dimerization constant ($K = [M]^2/[D]$) for gramicidin in phosphatidylcholine bilayers is $\sim 2*10^{-14}$ mol/cm^2 (Veatch et al., 1975), where $[M]$ and $[D]$ denote the monomer and dimer concentrations, respectively. The total peptide concentration ($[P]$) is given by $[M] + 2*[D]$; so $[M] = [D]$ ($= K$), when $[P] = 3*K$, or $\sim 10^{-13}$ mol/cm^2 for [Val^1]gramicidin A, which corresponds to a peptide/lipid ratio $\sim 10^{-3}$ (the surface area/phospholipid molecule is ~0.6 nm^2 [Tanford, 1980]). It would be necessary to work at even lower peptide/lipid ratios to resolve whether there are non-$\beta^{6.3}$-helical monomers in the membrane. Given the difficulties involved in obtaining CD spectra at peptide/lipd ratios below $\sim 3*10^{-3}$ (Wallace, 1986), it will be necessary to modify the peptide or the membrane environment (cf. Wallace et al., 1981; Killian et al., 1988*b*), such as to increase K by two orders of magnitude, or so.

Alteration of channel behavior by added solvent

There is no solvent history effect on gramicidin channels, but one cannot neglect the solvents. When present in the aqueous solution during the measurements, some solvents produce substantial changes in channel behavior (Fig. 1; Tables 1 and 2): benzene, chloroform, and trifluoroethanol. This is of interest because of the general anesthetic properties of some of these solvents (e.g., Seeman, 1972). That ethanol and methanol had no effect on the behavior of gramicidin channels is consistent with the lower potency of ethanol and methanol as anesthetic agents. Benzene, chloroform, and trifluoroethanol have lower water solubilities than the other solvents we used (Krieger, 1984). When added to the aqueous phase, these three solvents may thus partition better into the membrane than the other solvents and be more likely to alter the membrane's bulk properties: thickness, surface tension, dielectric constant, and interfacial dipole potential. Changes in thickness and surface tension should alter the peptides' channel-forming potency and average duration (e.g., Hladky and Haydon, 1972; Elliott et al., 1983), whereas, changes in dielectric constant and dipole potential should alter the single-channel conductance (Jordan, 1981, 1984).

From the inverse relation between bilayer thickness and channel duration (Hladky and Haydon, 1972; Urry et al., 1975), the benzene- and chloroform-induced decreases in the average channel duration could be caused by increases in bilayer thickness of ~0.2 and ~0.6 nm for benzene and chloroform, respectively. For benzene, much larger thickness changes than 0.2 nm would be predicted using ideal solution theory and a membrane/electrolyte partition coefficient of $\sim 10^3$ (De Young and Dill, 1988). For chloroform, however, the partition coefficient is only ~10 (Lieb et al., 1982), and we cannot account for the duration decrease based solely on a change in membrane thickness.

Single-channel conductances do not seem to vary with membrane thickness (Hladky and Haydon, 1972). But polar solvents, e.g., chloroform and trifluoroethanol, could alter the membrane's dielectric constant or dipole potential and thus alter the channel's conductance (Jordan, 1981, 1984). Benzene, however, has no dipole moment and a dielectric constant that is very close to that of saturated hydrocarbons (2.3 vs. 2). One would thus expect that benzene should have no effect on the channels' conductance. Benzene could, however, alter the membrane's dipole potential indirectly: in sodium dodecyl sulfate micelles, benzene appears to localize to the polar environment of the headgroups (Mukerjee and Cardinal, 1978). Thus, if benzene were at a similar position in

phospholipid membranes, it would increase the area/phospholipid molecule, thereby decreasing the surface dipole potential (MacDonald and Simon, 1987).

To obtain more information about the mechanism(s) by which the solvents alter the single-channel conductance, we compared their effect on channels formed by [Val^1]gramicidin A and [F_3Val^1]gramicidin A. The conductance of [F_3Val^1]gramicidin A channels is much less than that of [Val^1]gramicidin A channels (Russell et al., 1986), which is attributed to an increased height of the central barrier for ion movement through the former channel type (Russell et al., 1986; Jakobsson and Chiu, 1987). The increased height of the central barrier in [F_3Val^1]gramicidin A channels should make them more sensitive to changes in the membrane's dielectric constant and dipole potential. This was observed with chloroform, which induced a threefold larger conductance change in [F_3Val^1]gramicidin A channels than [Val^1]gramicidin A channels, in accordance with the finding that chloroform increases the capacitance of planar lipid bilayers (Reyes and Latorre, 1979). Benzene and trifluoroethanol, however, produced similar conductance changes in the two channel types. We do not understand the basis for these changes.

In any case, these solvent-induced changes in channel behavior show that care must be taken to ensure that no perturbing solvent is present when examining the properties of gramicidin channels. In particular, while trifluoroethanol may be useful for spectroscopic studies, our results indicate that other solvents sould be used in functional studies. More generally, our results reinforce the conclusion reached by Killian and Urry (1988) that the most consistent channel behavior is observed when gramicidin is cosolubilized with phospholipid.

We thank Dr. Suzanne Scarlata for making the fluorescence measurements and Drs. Sid Simon, Antoinette Killian, Anne Maddock, and Murray Becker for helpful discussions.

This work was supported by United States Public Health Service through National Intitutes of Health grants GM 21342 and GM 34968. D. B. Sawyer was supported by the M.D/PhD. student fellowship program at Cornell University Medical College.

Received for publication 15 September 1989 and in final form 20 November 1989.

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