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# Conformation of gramicidin A channel in phospholipid vesicles: A $^{13}C$ and $^{19}F$ nuclear magnetic resonance study

(paramagnetic ion/spin labels/spin lattice relaxation times/transmembrane channel/membrane structure)

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*Contributed by Elkan R. Blout, May 8, 1979*

**ABSTRACT** **We have determined the conformation of the channel-forming polypeptide antibiotic gramicidin A in phosphatidylcholine vesicles by using $^{13}C$ and $^{19}F$ NMR spectroscopy. The models previously proposed for the conformation of the dimer channel differ in the surface localization of the $NH_2$ and COOH termini. We have incorporated specific $^{13}C$ and $^{19}F$ nuclei at both the $NH_2$, and COOH termini of gramicidin and have used $^{13}C$ and $^{19}F$ chemical shifts and spin lattice relaxation time measurements to determine the accessibility of these labels to three paramagnetic NMR probes—two in aqueous solution and one attached to a phosphatidylcholine fatty acid chain. All of our results indicate that the COOH terminus of gramicidin in the channel is located near the surface of the membrane and the $NH_2$ terminus is buried deep within the lipid bilayer. These findings strongly favor an $NH_2$-terminal to $NH_2$-terminal helical dimer as the major conformation for the gramicidin channel in phosphatidylcholine vesicles.**

Gramicidin A is a linear polypeptide antibiotic that facilitates the diffusion of monovalent cations across membranes (1–4) by forming transmembrane channels (4, 5), each of which is made up of two molecules of gramicidin (6–10). The amino acid sequence of valine gramicidin A (11) is:

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-$NHCH_2CH_2OH$.

Veatch *et al.* (7) demonstrated that, if all of the gramicidin in a planar bilayer membrane is dimerized, all of the gramicidin forms ion-conducting channels. Further, Veatch and Stryer (8) used fluorescence energy transfer to show that gramicidin is dimerized on phosphatidylcholine liposomes. Hence the study of gramicidin in phosphatidylcholine vesicles makes possible the detailed conformational study of an ion-selective transmembrane channel using spectroscopic techniques.

$^1H$ NMR spectra of gramicidin in perdeuterated synthetic phosphatidylcholine vesicles at 72°C have been reported (12). The indole protons of gramicidin were resolved from the lipid resonances. It was argued that these indole protons were moderately exposed to the solution, on the basis of their $Tm^{3+}$-induced chemical shift change; however, due to the lack of markers on or near the $NH_2$ terminus, specific conformational conclusions could not be drawn. It should be noted that the nominal $NH_2$ terminus is, in fact, formylated, and the nominal COOH terminus is, in fact, an ethanolamide.

Four classes of models have been proposed for the conformation of the dimer gramicidin transmembrane channel (Fig. 1). Model *A* is the $NH_2$-terminal to $NH_2$-terminal $\Pi_6(L,D)$ helical dimer model proposed by Urry *et al.* (9, 13). More recently, Bradley *et al.* (14) have considered the possibility of forming a COOH-terminal to COOH-terminal dimer (Fig. 1*B*) of their original $\Pi_6(L,D)$ helix. Model *C* is the antiparallel-$\beta$ double helix proposed by Veatch *et al.* (15) as one of the dimer conformations found for gramicidin in nonpolar organic solvents (15, 16). Model *D* is a parallel-$\beta$ double helix with both $NH_2$ termini at one end of the channel. A distinctive feature of both double-helical models is the presence of *both* the $NH_2$ and COOH termini at the surfaces of the membrane (Fig. 1 *C* and *D*). For the $NH_2$-terminal to $NH_2$-terminal dimer (Fig. 1*A*) *only* the COOH termini are on the surfaces, and for the COOH-terminal to COOH-terminal dimer (Fig. 1*B*) *only* the $NH_2$ termini are on the surface.

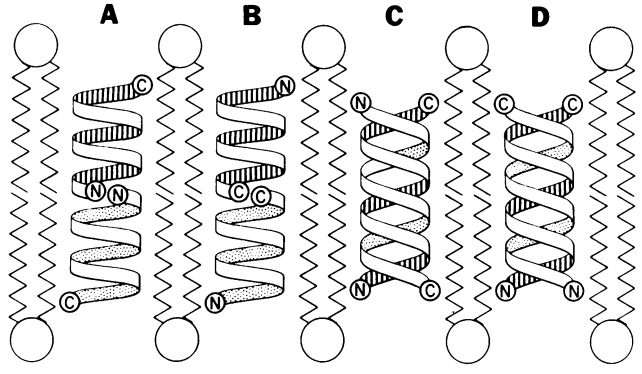


FIG. 1. Models proposed for the conformation of the gramicidin transmembrane channel. This diagram depicts a lipid bilayer membrane containing four different gramicidin dimer channel conformations. The gramicidin monomer polypeptide chain is represented as a ribbon with the $NH_2$ (N) and COOH (C) termini labeled. Note that one ribbon in each dimer has vertical stripes. All models are helical with roughly the same size hole down the middle through which ions and water may pass. Model *A* is the $NH_2$-terminal to $NH_2$-terminal $\Pi_6(L,D)$ model of Urry *et al.* (9, 13); model *B* is a COOH-terminal to COOH-terminal dimer of the same $\Pi_6(L,D)$ considered by Bradley *et al.* (14); models *C* and *D* are the antiparallel-$\beta$ and parallel-$\beta$ double helices of Veatch *et al.* (15), respectively. The results of this work strongly favor model *A* as the major conformation of the gramicidin channel in phosphatidylcholine vesicles.

We have incorporated specific $^{13}C$ and $^{19}F$ nuclei at both the $NH_2$ and COOH termini (Fig. 2) and have carried out NMR experiments to determine the relative accessibility of these $^{13}C$ and $^{19}F$ nuclei to paramagnetic NMR probes. Two of these probes are localized in the aqueous solution—manganous ion ($Mn^{2+}$) and thulium ion ($Tm^{3+}$)—and the third is localized in the membrane interior—a nitroxide spin label covalently at-

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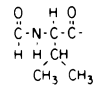
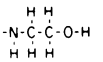
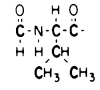
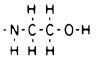
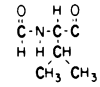
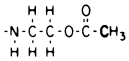
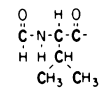
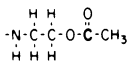
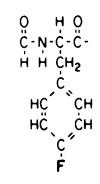
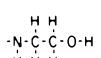
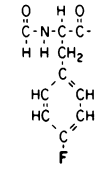
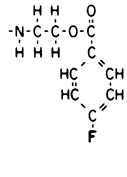
| Chemical name | $NH_2$ terminus | COOH terminus | Abbreviated name |
|---|---|---|---|
| A. Gramicidin |  |  | Gramicidin |
| B. [$^{13}$C-Formyl-Val$^1$]-gramicidin |  |  | *N*-[$^{13}$C]Gramicidin |
| C. [$^{13}$C-Formyl-Val$^1$]gramicidin, *O*-[*methyl*-$^{13}$C]acetyl |  |  | *N,C'*-[$^{13}$C]Gramicidin |
| D. [$^{13}$C-Formyl-Val$^1$]gramicidin, *O*-[*carbonyl*-$^{13}$C]acetyl |  |  | *N,C''*-[$^{13}$C]Gramicidin |
| E. [Formyl-$^{19}$F-Phe$^1$]gramicidin |  |  | *N*-$^{19}$F-Gramicidin |
| F. [Formyl-$^{19}$F-Phe$^1$]gramicidin, *O*-$^{19}$F-benzoyl |  |  | *N,C*-$^{19}$F-Gramicidin |

FIG. 2. Chemical structure and nomenclature of labeled gramicidin analogs and derivatives. The boldface **C** denotes specific $^{13}$C enrichment. Only the first amino acid residue is indicated at the $NH_2$ terminus, and only the ethanolamide and its esters on the COOH terminus. The intervening 14 amino acids are not shown.

tached near the end of a stearic acid chain of a phosphatidylcholine molecule. The large chemical shifts that occur for $^{13}$C (200 ppm for $^{13}$C relative to 10 ppm for $^1$H) and the low level of its natural abundance (1.1%) made it possible to enrich the gramicidin specifically (up to 80-fold) and to observe a number of the $^{13}$C-enriched gramicidin resonances in regions of the spectrum not obscured by the lipid resonances. Measurements on the $^{13}$C-enriched gramicidin and the $^{13}$C natural abundance lipid resonances were made simultaneously, thus providing a valuable internal control for the localization of the NMR probes. Because of the sensitivity of the $NH_2$ terminus of gramicidin to chemical modification (17, 18), $^{13}$C was incorporated into the $NH_2$ terminus with *no chemical change*. The *N*-formyl-L-valine-1 was removed and replaced with *N*-formyl- (90% $^{13}$C) L-valine (20% $^{13}$C) (designated *N*-[$^{13}$C]gramicidin, Fig. 2 B). The [$^{13}$C]valine-1 methyl groups proved to be a particularly good label, yielding an unobscured resonance much narrower than that of the [$^{13}$C]formyl carbonyl. A $^{13}$C label on the other end of the molecule was obtained by *O*-acetylating the hydroxyl of the COOH-terminal ethanolamide (designated *N,C*-[$^{13}$C]-gramicidin, Fig. 2 C and D), resulting in a derivative with activity on planar bilayer membranes *identical* to that of natural gramicidin. The *O*-acetyl-methyl (90% $^{13}$C) resonance is well resolved and is as large and sharp as some of the lipid resonances.

It was possible also to selectively insert $^{19}$F into gramicidin and observe the resonances without obstruction by other resonances, because membrane phospholipids contain no fluorine. *p*-Fluoro-L-phenylalanine was introduced as the $NH_2$-terminal amino acid (Fig. 2 E) and *p*-fluorobenzoyl was at the COOH terminus (designated *N,C*-$^{19}$F-gramicidin, Fig. 2 F), yielding a derivative with somewhat reduced activity on planar bilayer membranes.

The NMR parameters of interest for the experiments reported here are the chemical shift and the spin-lattice relaxation time, $T_1$, of the resonances. The unpaired electron of a paramagnetic atom provides a highly efficient additional dipole–dipole relaxation mechanism for any nucleus that comes within approximately 3–5 Å of it. The $T_1$ rate enhancement, $[(T_1)^{-1} - (T_{1o})^{-1}]$, in which $T_{1o}$ is the relaxation time without the paramagnetic atom, should fall off with an inverse sixth power dependence on the separation distance. $T_1$ can be measured for all of the resonances in a spectrum simultaneously.

## MATERIALS AND METHODS

**Preparation of Gramicidin Analogs and Derivatives.** The gramicidin obtained commercially (ICN) was crystallized once from ethanol. It contained 80% gramicidin A, 6% gramicidin B, and 14% gramicidin C by amino acid analysis and was used without further purification. Desformyl-gramicidin was prepared by a modification of the procedure of Sarges and Witkop (11) using Bio-Rad AG-MP-50 cation exchange resin. The $NH_2$-terminal amino acid (90% L-Val, 10% L-Ile) was removed by a modified Edman degradation. A formylated amino acid was then recoupled to the $NH_2$ terminus of the des[formyl-L-Val]gramicidin by using dicyclohexylcarbodiimide. The details of the chemical synthesis and characterization of these analogs will be published elsewhere. The COOH-terminal hydroxyl was acetylated by using a 4-fold excess of $^{13}$C-enriched acetic anhydride (Merck) in pyridine at 37°C for 12 hr. Similar conditions were used to react the *p*-fluorobenzoyl chloride with the COOH-terminal hydroxyl. For the *O*-acetyl derivative ≈90% of the label was attached to the COOH terminus and 10% to the tyrosine of gramicidin C. The purity of these analogs was assessed by NMR spectroscopy, amino acid analysis, thin-layer chromatography, and reverse phase high-pressure liquid chromatography.

**Membrane Activity of Derivatives and Analogs.** Planar lipid bilayer membranes made of glycerol 1-oleate and tetradecane were used to measure the conductance characteristics of the gramicidin analogs by the method of J. Morrow, W. Veatch, and L. Stryer (unpublished). The gramicidin analogs were added via the membrane-forming solution. The gross activity was measured as the conductance of a membrane made from a solution containing a given concentration of the analog divided by the conductance of a membrane made from a solution with the same concentration of natural gramicidin A.

**Preparation of Vesicles.** Dimyristoyl phosphatidylcholine (190 mg, Calbiochem) and gramicidin (15 mg) were dissolved in $CHCl_3$. The solvent was removed by evaporation, and the solutes were spread as a thin layer onto the walls of a 100-ml round-bottom flask and dried under reduced pressure for 1 hour. $^2H_2O$ (1.3 ml) was then added to the flask and the flask was incubated at 30°C for 20 min to hydrate the lipid. The sample was flushed with nitrogen and then sonicated for 0.5 hr at 37°C with a Braun-Sonic 1510 sonicator with a microtip probe. Salt solutions were added to the vesicle preparation after sonication and the sample was resonicated for 5 min at 37°C. All cations were added as the chloride salts. The concentrations of paramagnetic NMR probes used were: $MnCl_2$, 0.013 M; $TmCl_3$, 0.042 M; and lipid spin label, 2% of the total lipid. The gramicidin vesicles had the same D-[$^{14}$C]glucose trapped volume as control vesicles without gramicidin, but they did not trap $^{24}Na^+$.

**NMR Measurements.** NMR measurements were carried out

on a Brüker 270 NMR spectrometer, at a frequency of 68.7 MHz for $^{13}C$ and 254 MHz for $^{19}F$. All spectra were accumulated at 52°C in the pulsed Fourier transform mode, using quadrature detection and a 90° pulse unless otherwise indicated. The $T_1$ measurements were carried out using a (PD–180°–$\tau$–90°–AT) pulse sequence with a pulse delay, PD, of 2.7 sec. AT is the acquisition time. Typically, six values of the variable delay time $\tau$ were chosen. One very long final $\tau$ value (6.0 sec) made it possible to get a good estimate of the "infinite magnetization" for resonances with $T_1$ values less than 2.0 sec. Limited experiments with PD = 6 sec showed that this fast inversion recovery method gave accurate values for $T_1 \leq 2.0$ sec. The resonance amplitudes, $A$, were measured manually and fitted by a least-squares program to a general exponential $A(\tau) = A_\infty + (A_0 - A_\infty)e^{-\tau/T_1}$.

## RESULTS

The semisynthetic *N,C*-[$^{13}C$]gramicidin was identical in its planar lipid bilayer conductance activity to natural gramicidin. The *N,C*-$^{19}F$-gramicidin was about 15% as active as natural gramicidin as measured by the gross conductance (data not shown).

Three $^{13}C$-enriched gramicidin $^{13}C$ NMR resonances of *N,C'*-[$^{13}C$]gramicidin are well resolved from the lipid resonances: the Val-1 methyls, the *O*-acetyl methyl, and the formyl carbonyl (Fig. 3*A*). The $^{19}F$ NMR spectrum of *N,C*-$^{19}F$-gramicidin in lipid vesicles is given in Fig. 4*A*. In both cases the width of the resonances of gramicidin in the membrane is much greater than in the polar organic solvent dimethyl sulfoxide (see Figs. 3*B* and 4*B*). The most striking example is the [$^{13}C$]formyl carbonyl, which is 160 Hz (2.4 ppm) wide in the membrane, presumably due to its rigid attachment to the polypeptide backbone of the rigid channel structure. The [$^{13}C$]Val-1 methyls, also located at the $NH_2$ terminus (see Fig. 2 B), form a much narrower resonance (despite the splitting between the methyl groups) due to the rotation of these methyl groups. The *O*-[*methyl*-$^{13}C$]acetyl resonance is sharper still.

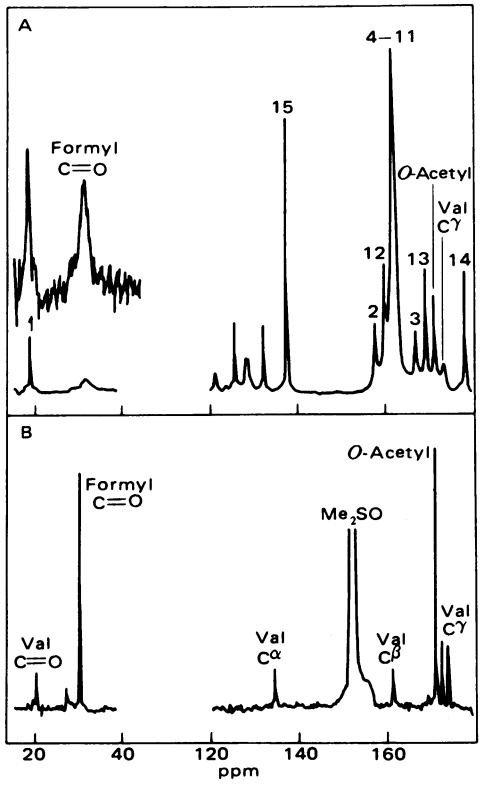



FIG. 3. $^{13}C$ NMR spectra of *N,C'*-[$^{13}C$]gramicidin in dimyristoyl phosphatidylcholine vesicles (*A*) and in perdeuterated dimethyl sulfoxide ($Me_2SO$) (*B*). The vesicle sample (*A*) contained 7.5 μmol of gramicidin at a lipid/peptide molar ratio of 30:1. The *N,C'*-[$^{13}C$]-gramicidin resonances are labeled, and the lipid resonances are numbered. Myristic acid resonances are numbered carbonyl carbon (1) through methyl carbon (14). The resonance of the choline methyls is numbered 15. The spectrum was accumulated for 2600 transients with a delay time of 6 sec at 52°C. (*Inset*) Region of the [$^{13}C$]formyl carbonyl expanded 8-fold vertically. The chemical shifts are relative to external carbon disulfide.

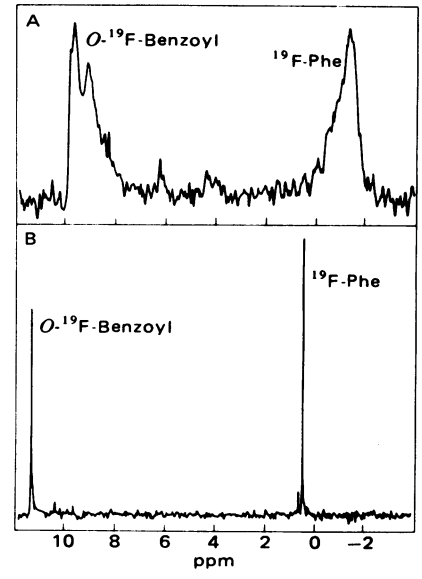



FIG. 4. $^{19}F$ NMR spectra of *N,C*-$^{19}F$-gramicidin in dimyristoyl phosphatidylcholine vesicles (*A*) and in perdeuterated dimethyl sulfoxide (*B*). The vesicle sample (*A*) contains 2 μmol of gramicidin analog at a lipid/peptide molar ratio of 30:1. The spectrum was accumulated at 50°C. The chemical shifts are relative to trifluoroacetic acid.

Table 1. NMR data summary

| Assignment | $Tm^{3+}$ chemical shift, ppm | $T_1$ rate enhancement, $sec^{-1}$: $Mn^{2+}$ | $T_1$ rate enhancement, $sec^{-1}$: Lipid spin label |
|---|---|---|---|
| [$^{13}C$]Phosphatidylcholine | | | |
| Choline methyl | 3.7 | 13 | 0.1 |
| Fatty acid carbons: | | | |
| $C_1$ | 0.2 | 14 | 0.2 |
| $C_2$ | 0.2 | 9 | 0.2 |
| $C_3$ | 0.4 | 3 | 1.1 |
| $C_4$–$C_{11}$ | ≃0.6 | 0.7 | 0.8 |
| $C_{12}$ | 0.2 | 0.1 | 0.6 |
| $C_{13}$ | 0.2 | 0.3 | 0.6 |
| $C_{14}$ | ≡0.0 | 0.3 | 0.8 |
| [$^{13}C$]Gramicidin | | | |
| COOH-terminal: | | | |
| *O*-Acetyl methyl | 2.1 | 1.9 | 0.0 |
| *O*-Acetyl carbonyl | 2.1 | | |
| $NH_2$-terminal: | | | |
| Val-1 methyl | 0.6 | 0.6 | 1.9 |
| Formyl carbonyl | 0.6 | 0.5 | 0.8 |
| $^{19}F$-Gramicidin | | | |
| COOH-terminal: | | | |
| *O*-$^{19}F$-Benzoyl | 0.80 | 23 | 3 |
| $NH_2$-terminal: | | | |
| $^{19}F$-Phe-1 | 0.08 | 1 | 9 |

**Thulium Ions Change Chemical Shifts of COOH-Terminal Labels Much More than Those of $NH_2$-Terminal Labels.** Thulium ($Tm^{3+}$) induced a large change in the chemical shift for the lipid choline methyl resonance (3.7 ppm) and very small changes in the carbonyl and other fatty acid resonances (Table 1). In the same experiment, the gramicidin COOH-terminal label (*O*-acetyl [$^{13}$C]methyl or [$^{13}$C]carbonyl) shifted substantially (2.1 ppm), whereas the $NH_2$-terminal $^{13}$C labels shifted only slightly (0.6 ppm). Similarly, the change in the $^{19}$F COOH-terminal label chemical shift was 10-fold greater than that of the $NH_2$-terminal label. Because the COOH-terminal ester groups would be expected to interfere with the intersubunit hydrogen bonding of the COOH-terminal to COOH-terminal helical dimer (Fig. 1*B*), measurements were carried out on gramicidin molecules without the COOH-terminal labels, and the same low chemical shift values were obtained for the $NH_2$-terminal labels. These data strongly imply that the COOH terminus is accessible on the surface of the membrane, and that the $NH_2$ terminus is not.

**Manganese Ions Increase Relaxation Rates of COOH-Terminal Labels Much More than Those of $NH_2$-Terminal Labels.** The spin-lattice relaxation times, $T_1$, for the lipid resonances and the [$^{13}$C]- and $^{19}$F-gramicidin resonances are given in the first column of Table 2 in the *absence* of any paramagnetic NMR probes. The relatively long $T_1$ values of the *O*-acetyl [$^{13}$C]methyl, [$^{13}$C]formyl carbonyl, and $^{19}$F-Phe-1 ($\approx$1 sec) make them very sensitive to the $T_1$ rate enhancements induced by paramagnetic NMR probes. The decreased $T_1$ values in the presence of about one $Mn^{2+}$ per lipid, as well as the $T_1$ rate enhancements, are given in Table 2. As found for $Tm^{3+}$, the effect of $Mn^{2+}$ on the lipid $^{13}$C resonances is greatest at the choline methyls (14 $sec^{-1}$) but appears to be substantial even for the first three carbons of the fatty acid chain (12–3 $sec^{-1}$). At the distal end of the chain, the values are relatively small but significant (0.3–0.6 $sec^{-1}$). The $T_1$ rate enhancement for the $NH_2$-terminal $^{13}$C labels (0.3–0.8 $sec^{-1}$) is comparable to that for the distal end of the fatty acid chain. The COOH-terminal *O*-acetyl [$^{13}$C]methyl had a substantially greater enhancement (1.9 $sec^{-1}$) than the $NH_2$-terminal labels. The COOH-terminal $^{19}$F label had a $T_1$ rate enhancement 20-fold greater than the $NH_2$-terminal $^{19}$F label. These low $NH_2$-terminal rate enhancements were also observed for the gramicidin molecules in which the COOH-terminal primary hydroxyl was underivatized (rightmost column of Table 2). Again, the COOH terminus appears to be accessible at the membrane surface and the $NH_2$ terminus does not.

**Lipid Spin Labels Increase Relaxation Rates of $NH_2$-Terminal Labels More than Those of COOH-Terminal Labels.** The third paramagnetic NMR probe is a nitroxide spin label attached to carbon 16 of a stearic acid molecule [2-(14-carboxytetradecyl)-2-ethyl-4,4′-dimethyl-3-oxazolidinyloxyl] that is in turn esterified specifically to position 1 of a phosphatidylcholine molecule. Myristic acid was esterified at position 2 in an effort to minimize the possibility of a lateral phase separation in the dimyristoyl phosphatidylcholine vesicles. As a control, the same concentration (2%) of 1-stearoyl-2-myristoyl phosphatidylcholine was used. The $T_1$ values for vesicles containing the spin-labeled lipid and for vesicles containing the control lipid, as well as the rate enhancements, are given in Table 3. It is evident that the spin-labeled lipid has its largest effects in the distal portion of the fatty acid chain. The enhancements near the surface are less and the value for the choline methyls actually was measured to be slightly negative. This localization is what one would expect from the chemical structure of the labeled lipid. The lipid probe caused its largest rate enhancement for the $NH_2$-terminal [$^{13}$C]Val-1 methyls (1.3 $sec^{-1}$) with essentially no effect on the COOH-terminal *O*-acetyl [$^{13}$C]methyl (0.0 $sec^{-1}$). The smaller value observed for the [$^{13}$C]formyl carbonyl may be due in part to its greater distance from the lipid spin label. The rate enhancement for the $NH_2$-terminal $^{19}$F label is 2-fold greater than that for the COOH-terminal label. Again, the same high $NH_2$-terminal rate enhancements were measured for gramicidin with the COOH-terminal hydroxyl unsubstituted (rightmost column of

Table 2. Effect of manganous ion on spin lattice relaxation rates

| Assignment | $T_1$, sec $Mg^{2+}$ | $T_1$, sec $Mn^{2+}$ | $T_1$ rate enhancement,* $sec^{-1}$ |
|---|---|---|---|
| [$^{13}$C]Phosphatidylcholine | | | |
| Choline methyl | 0.71 | 0.07 | 13 (15) |
| Fatty acid carbons: | | | |
| $C_1$ | 2.00 | 0.07 | 14 (10) |
| $C_2$ | 0.32 | 0.08 | 9 (6) |
| $C_3$ | 0.52 | 0.19 | 3 (3) |
| $C_4$–$C_{11}$ | 0.85 | 0.54 | 0.7 (1.1) |
| $C_{12}$ | 1.46 | 1.36 | 0.1 (0.8) |
| $C_{13}$ | ≥2.22 | 1.27 | 0.3 (0.6) |
| $C_{14}$ | ≥4.35 | 1.89 | 0.3 (0.3) |
| [$^{13}$C]Gramicidin | | | |
| COOH-terminal: | | | |
| *O*-Acetyl methyl | 1.10 | 0.36 | 1.9 |
| $NH_2$-terminal: | | | |
| Val-1 methyl | 0.58 | 0.43 | 0.6 (0.8) |
| Formyl carbonyl | 0.99 | 0.68 | 0.5 (0.3) |
| $^{19}$F-Gramicidin | | | |
| COOH-terminal: | | | |
| *O*-$^{19}$F-Benzoyl | 0.48 | 0.04 | 23 |
| $NH_2$-terminal: | | | |
| $^{19}$F-Phe-1 | 0.76† | 0.48† | 0.76† (0.1)† |

* Values in parentheses are for gramicidin analogs *without* COOH-terminal labels.
† At 40°C.

Table 3. Effect of nitroxide spin-labeled lipid on spin lattice relaxation rates

| Assignment | $T_1$, sec Control | $T_1$, sec Lipid spin label | $T_1$ rate enhancement,* $sec^{-1}$ |
|---|---|---|---|
| [$^{13}$C]Phosphatidylcholine | | | |
| Choline methyl | 0.61 | 0.79 | −0.4 (0.5) |
| Fatty acid carbons: | | | |
| $C_1$ | 1.86 | 1.39 | 0.2 (0.3) |
| $C_2$ | 0.29 | 0.28 | 0.1 (0.2) |
| $C_3$ | 0.41 | 0.40 | 0.1 (2.1) |
| $C_4$–$C_{11}$ | 0.69 | 0.53 | 0.4 (1.3) |
| $C_{12}$ | 1.19 | 0.75 | 0.5 (0.8) |
| $C_{13}$ | 1.70 | 1.27 | 0.2 (1.1) |
| $C_{14}$ | ≥3.34 | 1.61 | 0.3 (1.4) |
| [$^{13}$C]Gramicidin | | | |
| COOH-terminal: | | | |
| *O*-Acetyl methyl | 1.11 | 1.11 | 0 |
| $NH_2$-terminal: | | | |
| Val-1 methyl | 0.57 | 0.33 | 1.3 (2.5) |
| Formyl carbonyl | 1.12 | 0.91 | 0.2 (1.3) |
| $^{19}$F-Gramicidin | | | |
| COOH-terminal: | | | |
| *O*-$^{19}$F-Benzoyl | 0.49 | 0.21 | 3 |
| $NH_2$-terminal: | | | |
| $^{19}$F-Phe-1 | 0.61 | 0.12 | 6 (13) |

* Values in parentheses are for gramicidin analogs *without* COOH-terminal labels.

Table 3). These results imply that the $NH_2$-terminal label is accessible to the lipid probe, which lies in the interior of the membrane, but that the COOH-terminal label is not.

## DISCUSSION

The results with $Tm^{3+}$ and $Mn^{2+}$ demonstrate that the COOH terminus of gramicidin is accessible at the surface of the membrane, but that the $NH_2$ terminus is not. The lipid spin label results indicate that the $NH_2$ terminus is accessible deep in the membrane, but the COOH terminus is not. *These results exclude the COOH-terminal to COOH-terminal helical dimer (Fig. 1B) and the antiparallel-β and parallel-β double helices (Fig. 1 C and D) and strongly favor the $NH_2$-terminal to $NH_2$-terminal helical dimer (Fig. 1A) as the major conformation of the gramicidin channel in phosphatidylcholine vesicles.*

Urry *et al.* (9) and Bamberg and Janko (10) have both reported the synthesis of a covalent gramicidin dimer linked $NH_2$ terminus to $NH_2$ terminus that is highly active and has many of the conductance properties expected for such a covalent dimer. Such a linkage is not possible, in principle, for an antiparallel double helix. Apell *et al.* (19) have recently studied a gramicidin derivative with three negative charges on the COOH terminus. They observe that it is *not* active when added from one side of the membrane but is active when added to both sides. This result is not consistent with the parallel double helix as the *only* conformation. If these two derivatives have the same channel conformation, then both types of double helical structures are eliminated, leaving only the $NH_2$-terminal to $NH_2$-terminal dimer of helices (Fig. 1*A*) as a possible conformation. However, the presence of small amounts of a second channel conformation has been considered by Bradley *et al.* (14) on the basis of activity studies of gramicidin analogs with sterically bulky replacements for the *N*-formyl group. The interpretation of the present NMR study is *not dependent* upon the assumption that there is only one conformation, because the location of both ends of the molecule was determined for the same molecules in a single experiment. It may well be that small amounts (<20%) of double helices or other conformations may coexist with the major $NH_2$-terminal to $NH_2$-terminal helical dimer. The question of possible channel heterogeneity remains to be resolved.

We need no longer be content to study only the effect of a polypeptide on the NMR spectrum of the lipid; it is now possible to study membrane-bound peptides directly with NMR techniques (20), even a transmembrane channel such as gramicidin. Increases in sensitivity can be obtained with more sophisticated NMR instrumentation and the use of specific enrichment with $^{13}C$-containing amino acids. These results suggest that NMR techniques will be particularly useful for determining which regions of the polypeptide chain of a membrane protein lie within the membrane interior and which are outside the membrane. These techniques are well suited for studying the "hydrophobic peptide" region of glycophorin (the major human erythrocyte membrane sialoglycoprotein).

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