Stabilizing Effect of D-Alanine[2] in Gramicidin Channels†

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ABSTRACT: We have investigated the effects of replacing Gly^2 by D-Ala^2 in gramicidin A (gA) analogues that have either L-Val, L-Ala, or Gly as the formyl-N-terminal residue. Circular dichroism, two-dimensional nuclear magnetic resonance, and hybrid channel experiments all show that [Ala^1,D-Ala^2]gA channels are structurally equivalent to the native [Val^1,Gly^2]gA channels, being formyl-NH-to-formyl-NH dimers of single-stranded, right-handed $\beta^{6.3}$ helices. Replacing the Val^1 of gA by Ala or Gly decreases the average channel duration. Replacing Gly^2 by D-Ala in [Val^1,Gly^2]gA increases the average channel duration 4-fold and the single-channel conductance by ~15%; replacing Gly^2 with D-Ala in [Ala^1,Gly^2]gA or [Gly^1,Gly^2]-gA leads in each case to a 10-fold increase in the average channel duration with only modest changes in the single-channel conductance, which depends on the identity of the position-one residue and the permeant ion. These results illustrate the importance of neighboring-residue side chain and backbone interactions for the modulation of channel properties.

A general question for ion channels and other membrane and soluble proteins is how the primary sequence affects the structure of the protein and thereby its function. One important component of this overall problem is how different amino acids interact and how the effects of individual mutations depend upon the "context" of other amino acids in the sequence. In this article we focus on changes at positions one and two in the primary sequence of the channel-forming gramicidins.

The linear gramicidins are a family of hydrophobic pentadecapeptides produced by *Bacillus brevis* (Dubos, 1939), which form membrane-spanning cation-specific channels by the joining of two monomers into a head-to-head (formyl-NH-to-formyl-NH) manner (O'Connell et al., 1990). Engineered sets of linear gramicidins are good models for investigating the effects of sequence substitutions, because of the large amount of detailed structural and functional information that is available from previous studies. The basic channel structure is known from NMR spectroscopy (Arseniev et al., 1986; Smith et al., 1990; Ketchem et al., 1993; Hu et al., 1993; Koeppe et al., 1994a), and the primary sequence can be altered by peptide chemical methods, which allow for the convenient incorporation of nongenetic amino acids. The primary sequences of [Val^1,Gly^2] gramicidin A (gA)[1] (Sarges & Witkop, 1965) and the analogues used in this study are given in Table 1.

Substitutions at the first position of [Val^1,Gly^2]gA, where the two monomers join to form the membrane-spanning dimer, can have large effects on the single-channel conductance and the average channel duration (Mazet et al., 1984; Russell et al., 1986; Durkin et al., 1990). Position-one substitutions alter channel function predominantly through electrostatic interactions between the passing ions and the dipole of the side chain (Koeppe et al., 1990). These ion−dipole interactions can be large in magnitude and extend over considerable distances (1−2 nm) because of the low dielectric constant within the membrane. The duration (τ) of a "mutant" channel is related to the geometry of the first side chain, with τ increasing as Gly^1 < straight-chain[1] < γ-branch[1] < β-branch[1]. For this series of analogues, the results suggest that side chain−backbone interactions are important (Koeppe & Andersen, 1993).

To more fully investigate this possibility, the first two amino acids from the amino terminus of [Val^1,Gly^2]gA were replaced with glycine and alanine to determine the effects on channel structure and function. This allowed us to test the effects of changing the small, flexible amino acid Gly at position two as conservatively as possible, to D-Ala, which keeps the strictly alternating L,D sequence of natural [Val^1,Gly^2]-gA. The structure and function of the mutant channels were examined by CD, NMR, and single-channel analysis and compared to [Val^1,Gly^2]gA.

MATERIALS AND METHODS

Materials. Gramicidin D, the naturally occurring mixture of linear gramicidins, and L-Ala were from Sigma Chemical Co. (St. Louis, MO). [Val^1,Gly^2]gA was isolated as in Koeppe et al. (1985). DMF, ethyl acetate, ethanol, formic acid, and acetic anhydride were from Aldrich Chemical Co. (Milwaukee, WI). DMPC and DPhPC were from Avanti Polar Lipids (Alabaster, AL). The DPhPC was further

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[1] Abbreviations: gA, gramicidin A; CD, circular dichroism; RH, right-handed; LH, left-handed; NMM, *N*-methylmorpholine; DMF, dimethylformamide; TFE, trifluoroethanol; MeOH, methanol; DMPC, dimyristoylphosphatidylcholine; DPhPC, diphytanoylphosphatidylcholine; ABI, Applied Biosystems Inc. (a division of Perkin-Elmer); HPLC, high-pressure liquid chromatography; HMP, *p*-hydroxymethylphenoxyacetic acid; SDS, sodium dodecyl sulfate; COSY, correlated spectroscopy; NOESY, nuclear Overhauser enhancement spectroscopy.

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Table 1: Amino Acid Sequences of Gramicidin A Analogues

Analogue (abbrev.)	Sequence
Gramicidin A: (gA)	L L D L D L D L D L D L D L HCO-Val-Gly-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
[Gly^1,Gly^2]gA:	L D L D L D L D L D L D L D L HCO-**Gly**-Gly-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
[Ala^1,Gly^2]gA:	L L D L D L D L D L D L D L D L HCO-**Ala**-Gly-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
[Val^1,D-Ala^2]gA:	L D L D L D L D L D L D L D L HCO-Val-**Ala**-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
[Gly^1,D-Ala^2]gA:	D L D L D L D L D L D L D L D L HCO-**Gly**-**Ala**-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
[Ala^1,D-Ala^2]gA:	L D L D L D L D L D L D L D L D L HCO-**Ala**-**Ala**-Ala-Leu-Ala-Val-Val-Val-Trp-Leu-Trp-Leu-Trp-Leu-Trp-$NHCH_2CH_2OH$ 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

cleaned by ion exchange chromatography as described in Andersen (1983). *n*-Decane was 99.9% pure from Wiley Organics (Columbus, OH). NaCl was Suprapur grade from E. Merck, Darmstadt (through MCB, Cincinnati, OH). Before use, it was roasted for at least 24 h at 550−600 °C and stored in an evacuated desiccator over NaOH. Ethanol used in dilutions for single-channel studies was from U.S. Industrial Chemicals (Tuscola, IL).

Ethanolamine was from Aldrich Chemical Co. (Milwaukee, WI). It was distilled in vacuo, sealed in glass, and stored at −20 °C until use. HMP resin and solvents for solid-phase peptide synthesis (piperidine, dichloromethane, dicyclohexylcarbodiimide, 1-hydroxybenzotriazide hydrate, and dimethylaminopyridine) were from ABI (Foster City, CA). *N*-Methylpyrrolidone was from ABI or Baxter (McGaw Park, IL). Fmoc amino acids were from Bachem (Philadelphia, PA) or Peninsula Labs (Belmont, CA).

Methanol (HPLC grade) was from Baker (Muskegon, MI) or Chempure (Houston, TX). A prepacked 4.6 × 250 mm column of Zorbax-C8, a 5 μm octylsilica reversed-phase spherical packing, was from Dupont Corp. (Wilmington, DE). All other chemicals were reagent grade.

Synthesis of Analogues. [Gly^1,Gly^2]gA, [Gly^1,D-Ala^2]gA, and [Ala^1,Gly^2]gA were produced by semisynthesis as described in Weiss and Koeppe (1985). All other analogues were synthesized by loading an HMP resin with Fmoc-L-Trp and then deprotecting and extending the loaded resin using an ABI model 431A peptide synthesizer to add the appropriate Fmoc amino acid precursors, followed by formyl-L-Ala or formyl-L-Val at the last step. These amino acids were formylated as described in Weiss and Koeppe (1985).

The products from the total synthesis were released from the resin by ethanolaminolysis in anhydrous ethanolamine/DMF (50/50) at 60 °C for 15−24 h. After filtering of the resin, each product was precipitated by the addition of water, collected by centrifugation, and resuspended (3×). After the final precipitation the pellet was dried, resuspended in methanol, and purified on a phenyl preparative column (Koeppe et al., 1985). Aliquots were twice purified and diluted into 10−100 volumes of ethanol to give a final concentration of 10^{-8}−10^{-9} M of the stock solution used for the single-channel experiments.

CD Measurements. [Val^1,Gly^2]gA and [Ala^1,D-Ala^2]gA in DMPC (160−190 μM gramicidin, 4.8−5.3 mM lipid) were prepared as small unilamellar vesicles for CD spectroscopy as described by Sawyer et al. (1990). [Val^1,Gly^2]-gA in SDS (900 μM gramicidin, 45 mM SDS) was prepared as described by Arseniev et al. (1985) with the following modifications: The SDS stock solution was sonicated at 60 °C for 10 min using a Branson W-185 Cell Disruptor (level 5) fitted with a model 431-A cup horn accessory (Branson Sonic Power Co., Danbury, CT) before adding it to the TFE-dissolved [Val^1,Gly^2]gA. The final solution was sonicated as above for 40 min at 45 °C and then centrifuged at 40 °C. After centrifugation, the supernatant was transferred to a fresh 1.5-mL tube and recentrifuged at 40 °C. The final, clear solution was stored at 40 °C. A small aliquot of the sample was diluted in methanol and the concentration determined from the absorbance at 280 nm. The [Ala^1,D-Ala^2]gA in SDS (700 μM gramicidin, 70 mM SDS) was from the NMR preparation (see below).

CD spectra were recorded on a Jasco J-710 spectrometer. A total of 12 scans were accumulated for each sample at a speed of 50 nm/min using either a 0.1 or 0.01 cm path length cell.

NMR Spectroscopy. The procedure was adapted from Arseniev et al. (1985). Six milligrams (3.2 μmol) of [Ala^1,D-Ala^2]gA were vacuum dried overnight (0.1 mm Hg, liquid N_2 trap) and dissolved in 0.1 mL of TFE-d_3. A preformed micelle solution was prepared by dissolving 92 mg (320

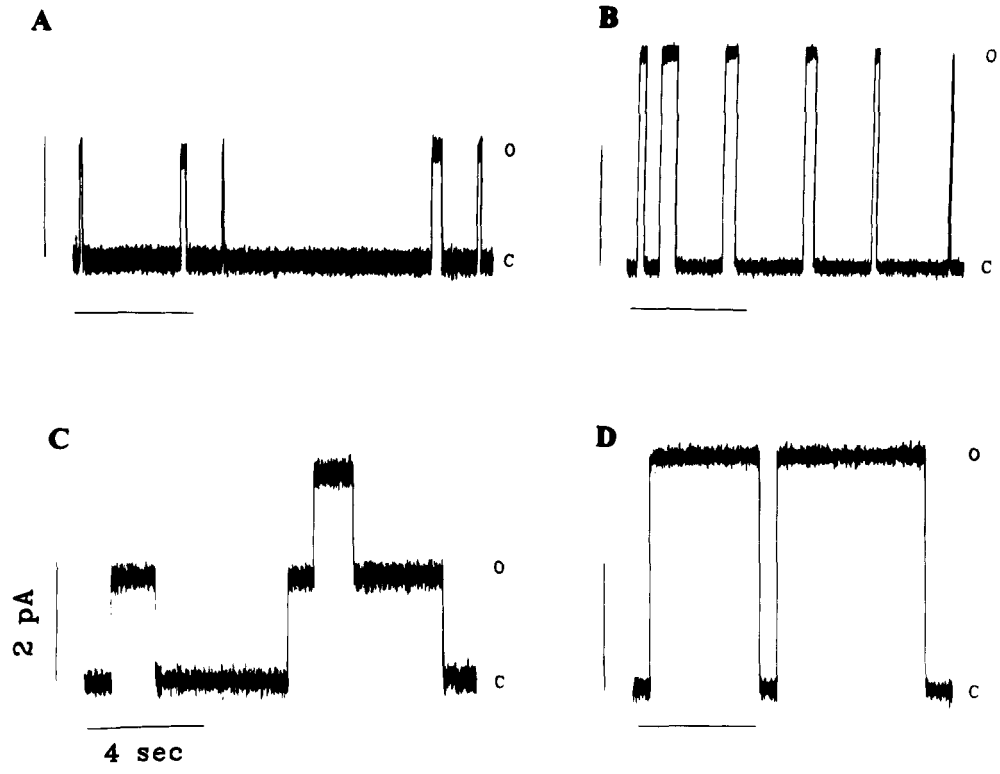

FIGURE 1: Single-channel current traces seen with the four Ala ↔ Gly gramicidin A analogues. (A) [Gly¹,Gly²]gA channels; (B) [Ala¹-Gly²]gA channels; (C) [Gly¹,D-Ala²]gA channels; (D) [Ala¹,D-Ala²]gA channels. The current traces are shown using identical current and time scales. The calibration bars denote 2 pA (vertically) and 4 s (horizontally). Channel appearances are upward; the letters "c" and "o" to the right of each trace denote the current levels corresponding to no channels (closed) and to one conducting channel (open), respectively. Conditions: 200 mV, 1.0 M NaCl, 100 Hz filter.

μmol) of SDS-d_{25} in 0.7 mL of H_2O and 0.1 mL of D_2O (N_2-purged), vortexing, and then sonicating at 55 °C for 10 min using power level 5 of a Branson W-185 Cell Disrupter fitted with a model 431-A cup horn accessory. The pH was adjusted to 6.7 ± 0.2 with 10 mM HCl, and the micelle solution was stored at 55 °C. Aliquots (20 μL) of the warmed solution of [Ala¹,D-Ala²]gA in TFE-d_3 were added to the preformed solution of SDS-d_{25}, with vortexing and sonication (as above) between successive additions. The final dispersion was sonicated 1 h, as above, and centrifuged 10 min at 14000g. The clear supernatant (containing 2.4 mM [Ala¹,D-Ala²]gA) was transferred to a warmed round-bottom NMR tube and maintained at 50−55 °C in the dark.

Double quantum-filtered COSY spectra (Rance et al., 1983) were recorded at 55 °C using a 500 MHz Varian VXR 500S spectrometer over a sweep width of 6 kHz. A total of 512 FID of 4096 complex points were acquired with 32 scans per t_1 value. The spectra were zero-filled to a 4K × 4K matrix and processed on a Sun 4/110 computer using VNMR software (Varian). NOESY spectra were recorded at mixing times of 40 and 100 ms, collected with 512 t_1 points and 4096 complex t_2 points with 64 scans per t_1 value. Spectral solvent suppression was accomplished by presaturation.

Single-Channel Measurements. Planar bilayers were formed from DPhPC in n-decane (2−3% w/v) at 25 ± 1 °C. Single-channel measurements were done using the bilayer punch method (Andersen, 1983) at an applied potential of 200 mV. The electrolyte solutions were unbuffered 1.0 M NaCl or CsCl, made the day of the experiment. Small aliquots of gramicidin in absolute ethanol were added to both halves of the chamber to produce about 1 channel event/s. Channel activity was monitored as discrete rectangular current jumps. The data analysis was as described by Sawyer et al. (1989) and Durkin et al. (1990).

RESULTS

Channel Activity. The functional characteristics of gramicidin channels with Gly or Ala at positions one and two were examined by single-channel experiments and compared to native gA. Single-channel current traces obtained with [Gly¹,Gly²]gA, [Ala¹,Gly²]gA, [Gly¹,D-Ala²]gA, and [Ala¹,D-Ala²]gA channels in 1.0 M NaCl are shown in Figure 1. There is considerable variation in conductance and duration among the channels. Importantly, to a first approximation, the effect of Gly → Ala exchanges at positions one and two are independent of one another.

Conductances of Analogue Channels. Single-channel experiments were done with both Cs^+ and Na^+ as permeant ions. Figure 2 summarizes the conductance results obtained with 1.0 M NaCl. The narrow, well-defined peaks in the current transition amplitude histograms indicate that each analogue forms only a single channel type (as is also true with [Val¹,Gly²]gA and [Val¹,D-Ala²]gA channels, see below). The conductance is more sensitive to the identity of the first amino acid than to the second; the change of Gly² → D-Ala² causes little change in the conductance. The single-channel conductances and average durations obtained with Na^+ are summarized in Table 2.

Table 3 summarizes the results of the single-channel experiments in 1.0 M CsCl. While the general trends are similar, there are differences between Cs^+ and Na^+ permeation. Compared to [Val¹,Gly²]gA channels, Ala¹ or D-Ala² substitutions decrease the Cs^+ conductance, whereas these substitutions increase the Na^+ conductance. A Val¹ → Gly¹

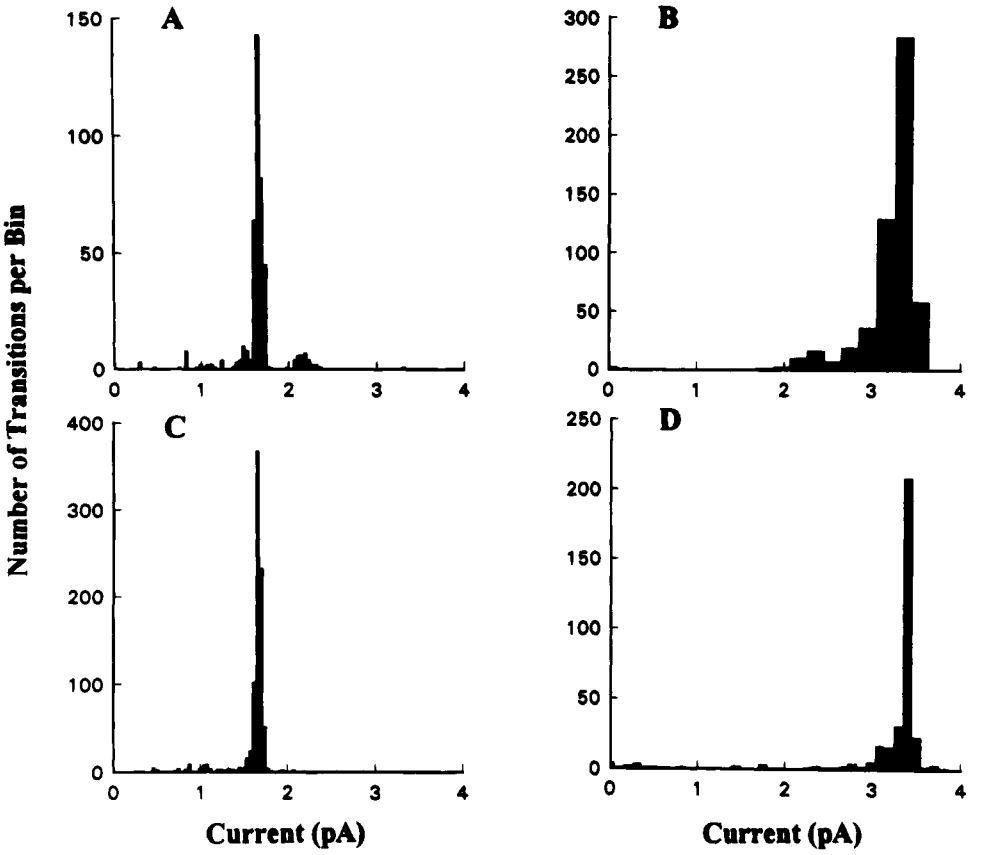

FIGURE 2: Current transition amplitude histograms for the four Ala ↔ Gly gA analogues. (A) [Gly^1,Gly^2]gA channels; (B) [Ala^1,Gly^2]gA channels; (C) [Gly^1,D-Ala^2]gA channels; (D) [Ala^1,D-Ala^2]gA channels. The average single-channel conductances are listed in Table 2. Conditions: 200 mV, 1.0 M NaCl.

Table 2: Single-Channel Durations and Conductances in 1.0 M NaCl

	position 2			
	A τ (ms)		B g (pS)	
position 1	Gly	D-Ala	Gly	D-Ala
L-Val	800	3300	14.6	16.7
Gly	70	1100	8.3	8.3
L-Ala	190	2200	16.4	17.0

Table 3: Single-Channel Durations and Conductances in 1.0 M CsCl

	position 2			
	A τ (ms)		B g (pS)	
position 1	Gly	D-Ala	Gly	D-Ala
L-Val	800	2600	47.2	40.6
Gly	90	1300	32.2	31.9
L-Ala	200	2400	45.4	42.2

substitution causes a smaller relative decrease in Cs^+ conductance than in Na^+ conductance, and the decrease is again independent of whether Gly or Ala is at position two. There is a differential effect on the Cs^+/Na^+ ion selectivity among the channels formed by these analogues.

Durations of Analogue Channels. Gly → Ala substitutions at position one and/or two have larger effects on the average channel durations than on the conductances. The single-channel duration distributions (Figure 3) are well described by single-exponential distributions, with average durations varying between 70 ms for [Gly^1,Gly^2]gA channels and 2200 ms for [Ala^1,D-Ala^2]gA channels (Tables 2 and 3). The substitution of the isopropyl side chain of Val^1 with the much smaller methyl side chain of Ala decreases the average channel duration by 75% (Figure 3A). Continuing the reduction of the side chain size down to the hydrogen of Gly dramatically decreases the (original) duration by 90% (Figure 3A). The substitution of D-Ala at the second residue of [Gly^1,Gly^2]gA or [Ala^1,Gly^2]gA in both cases increases the duration. [Gly^1,D-Ala^2]gA channels have a 16-fold longer average duration than [Gly^1,Gly^2]gA channels. Similarly, [Ala^1,D-Ala^2]gA channels have a 12-fold longer average duration than [Ala^1,Gly^2]gA channels (Table 2). Longer channel durations are promoted by the presence of the more hydrophobic and larger Ala at position two, with smaller changes at position one.

In CsCl, the durations follow similar trends and present few surprises (Table 3). For Gly or Ala at positions one and/or two, the average duration does not depend on the nature of the permeant ion. The D-Ala^2 side chain promotes important favorable interactions between the two monomers that stabilize the channel. The case of [Val^1, D-Ala^2]gA will be addressed below.

Structural Studies. The secondary structure of [Ala^1,D-Ala^2]gA in DMPC and in SDS was investigated by CD spectroscopy, and was more fully examined using 2-D ^{1}H NMR spectroscopy in SDS-d_{25} micelles. Both methods show that the analogue retains the RH $\beta^{6.3}$-helical structure of [Val^1,Gly^2]gA channels.

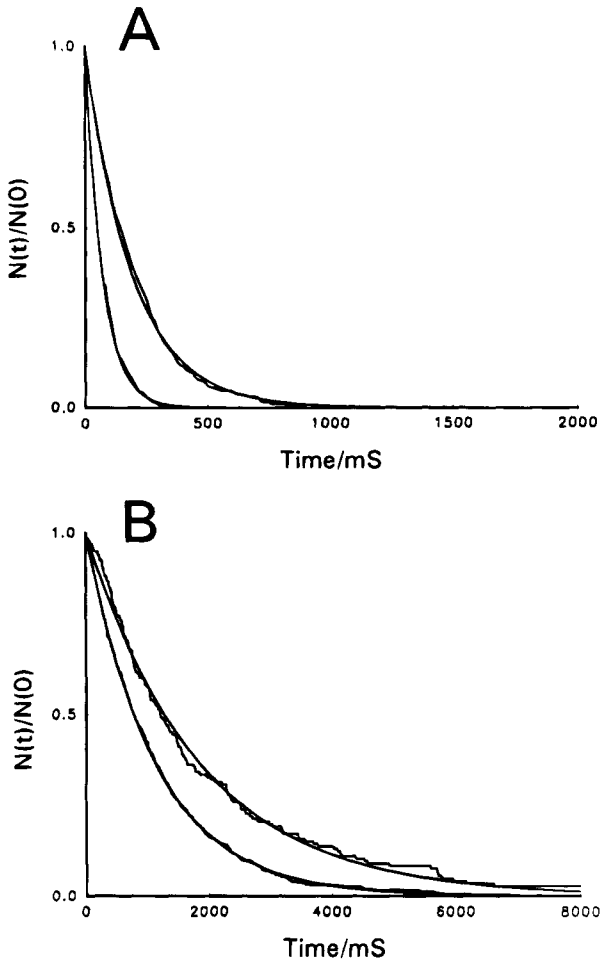

FIGURE 3: Duration distributions for the four Ala ↔ Gly gA analogues. (A) [Gly¹,Gly²]gA channels (τ = 70 ms) and [Ala¹,Gly²]-gA channels (τ = 190 ms); (B) [Gly¹,D-Ala²]gA channels (τ = 1100 ms) and [Ala¹,D-Ala²]gA channels (τ = 2200 ms). The results are displayed as normalized survivor plots. Note that the abscissa in panel A (0−2000 ms) is shorter than that in panel B (0−8000 ms). The continuous curves show the fits of single-exponential distributions to the results. Conditions: 200 mV, 1.0 M NaCl.

Circular Dichroism Spectroscopy

DMPC Environment. The CD spectrum of [Val¹,Gly²]gA in DMPC vesicles (Figure 4) is representative of the single-stranded, RH $\beta^{6.3}$-helical "channel" structure (Wallace et al., 1981; Killian et al., 1988). This spectrum is characterized by positive peaks at ~218 and ~235 nm, separated by a valley at ~230 nm. The depth of the valley has been suggested to indicate the amount of double-stranded helix present in a membrane or solution (Bañó et al., 1991, 1992). The CD spectrum of [Ala¹,D-Ala²]gA in DMPC vesicles is similar to the [Val¹,Gly²]gA spectrum with only slightly less intense peaks. This similarity in the spectra shows that the major conformer for both gramicidins in DMPC is the single-stranded RH $\beta^{6.3}$ helix.

Heating sometimes alters the mix of gramicidin conformers in a sample (Masotti et al., 1980; Shungu et al., 1986; Killian et al., 1988). In the present case, an overnight heating of the [Ala¹,D-Ala²]gA at 65 °C did not significantly affect the spectrum (results not shown).

SDS Environment. The CD spectra of [Val¹,Gly²]gA and [Ala¹,D-Ala²]gA were also obtained in an aqueous SDS micelle environment because the spectrum of [Val¹,Gly²]gA in this environment resembles its DMPC vesicle spectrum

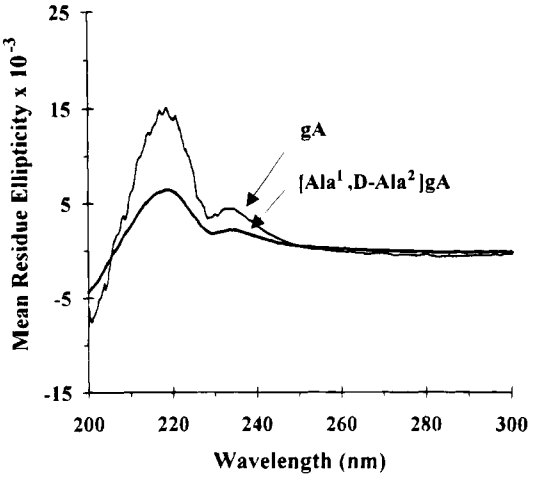

FIGURE 4: CD spectra of [Val¹,Gly²]gA, and [Ala¹,D-Ala²]gA in DMPC (gramicidin/lipid mole ratio = 1/30; 25 °C). Both of the spectra are indicative of a RH $\beta^{6.3}$-helical conformation.

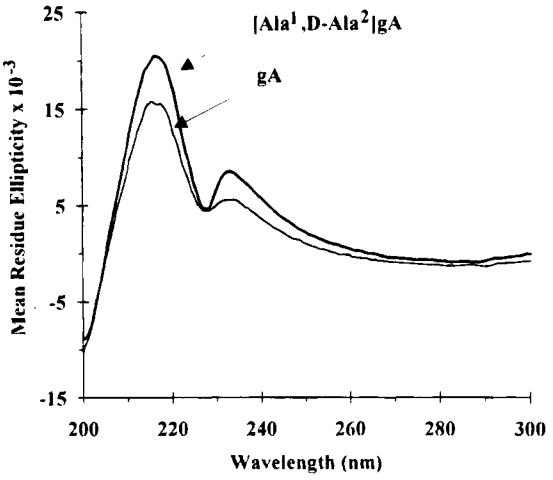

FIGURE 5: CD spectra of [Val¹,Gly²]gA and [Ala¹,D-Ala²]gA dispersed in SDS ([Val¹,Gly²]gA/SDS mole ratio = 1/50; [Ala¹,D-Ala²]gA /SDS mole ratio = 1/100; 25 °C).

and because this lipid-like environment is suitable for NMR spectroscopy (Arseniev et al., 1985, 1986; Killian et al., 1994; Koeppe et al., 1994b). Compared to the spectrum in DMPC, the [Val¹,Gly²]gA spectrum in SDS retains the same general shape, but the peaks are of greater positive intensity (Figure 5). Likewise, the [Ala¹,D-Ala²]gA spectrum resembles the general [Val¹,Gly²]gA pattern but exhibits peak intensities that surpass even those of [Val¹,Gly²]gA in SDS.

Heating the SDS samples had little effect on the general shape of the CD spectra of either [Val¹,Gly²]gA or [Ala¹,D-Ala²]gA (results not shown).

2-D NMR Spectroscopy

[Ala¹,D-Ala²]gA in SDS micelles was investigated using 2-D ^{1}H NMR. Figure 6 shows an overlay of the CαH−NH "fingerprint" regions of NOESY and DQCOSY spectra, with the COSY quartets numbered according to the amino acid sequence of [Ala¹,D-Ala²]gA. From the overlay, the sequential NOE interactions between adjacent residues are evident (Wüthrich, 1986), as are the NOE peaks that indicate a RH $\beta^{6.3}$ helix. These RH helix peaks (marked with dashed circles in Figure 6) show the $NH_i-C\alpha H_{i+6}$ interactions for even i and $NH_i-C\alpha H_{i-6}$ interactions for odd i (Arseniev et al., 1985).

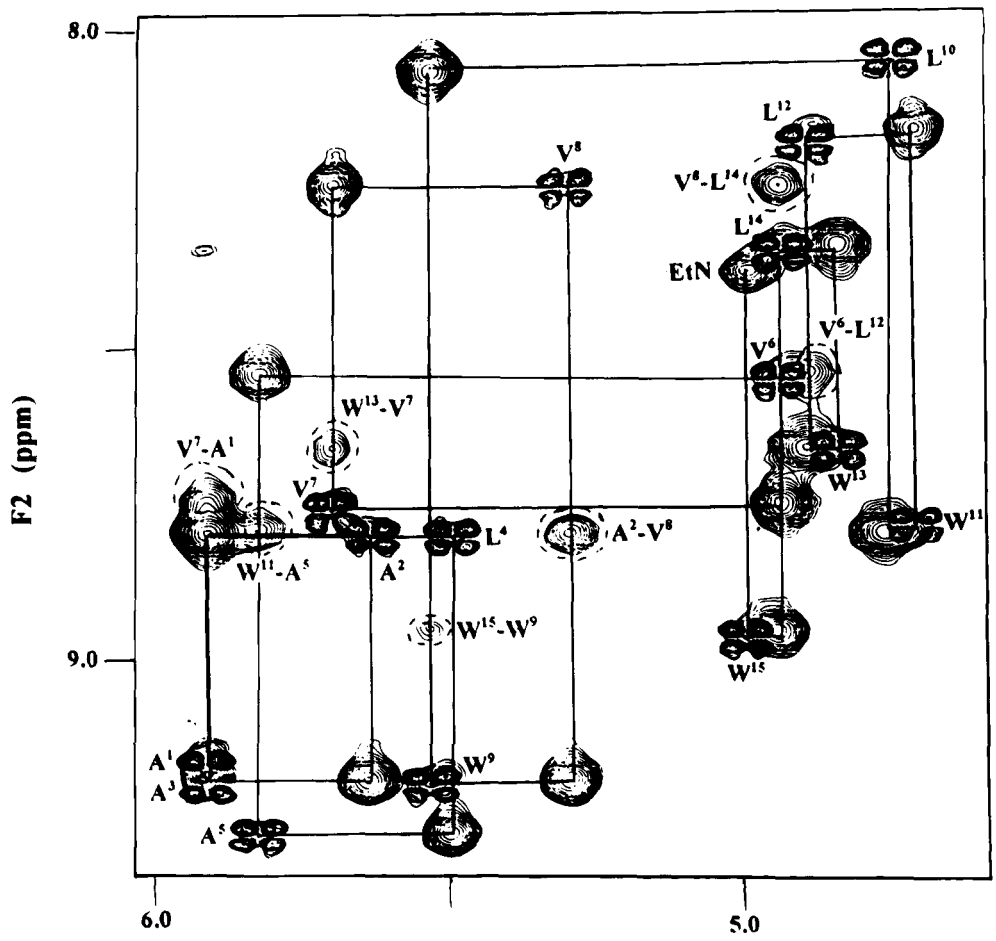

FIGURE 6: Overlay of the fingerprint regions of DQCOSY and NOESY (40 ms mixing time) spectra of [Ala[1],D-Ala[2]]gA in SDS, 55 °C. The COSY quartets are labeled with the one-letter amino acid abbreviations and numbered according to the sequence. The COSY peaks of L^{10}, L^{12}, L^{14}, V^6, W^{13}, and W^{11} (residues distant from the substitutions) form a "crescent" arc which is similar to that of the gramicidin A COSY spectrum. The sequence is traced as in Wüthrich (1986), from A^1, through alternating COSY and NOESY peaks, to W^{15} and finally the W^{15}—ethanolamine NOESY peak (labeled EtN). The NOESY peaks due to the RH $\beta^{6.3}$ helix are labeled and enclosed by dashed circles.

In the fingerprint region, two new alanine cross peaks are located at (9.18, 5.92) and (8.81, 5.64) ppm for (NH, CαH) of Ala^1 and D-Ala^2, respectively. The Ala^1 cross peak overlaps that of Ala^3, and the D-Ala^2 cross peak corresponds very closely to one of the Gly^2 cross peaks of [Val^1,Gly^2]-gA. The series of quartets for $L^{10}...L^{12}...L^{14}...V^6...W^{13}...W^{11}$ define a characteristic "crescent" arc that is similar to that in the [Val^1,Gly^2]gA COSY spectrum.

Because of the changes in local environment caused by the two amino acid substitutions, the chemical shifts of some nuclei of amino acids that are distant in the primary sequence are different from those in [Val^1,Gly^2]gA. In the fingerprint region, which defines the backbone and thus the positions of the carbonyl oxygens, the Trp^9 backbone NH and the Val^7 CαH (about one helical turn away from the position of the alanine substitutions), show the largest shifts, moving downfield by more than 0.2 ppm, followed by the Trp^{15} backbone NH (two helical turns away from the substitutions), which has shifted downfield by more than 0.15 ppm. These changes may indicate that some of the altered channel properties could result from subtle long-range adjustments of the backbone.

Leu^{10} is about 1.5 helical turns away from the sites of the substitutions. The environment of the Leu^{10} backbone and side chain remain essentially unchanged (Figure 7). As in [Val^1,Gly^2]gA, the side chain of Leu^{10} is strongly shielded by the indole ring of Trp^9 (Koeppe et al., 1995). The shielding is shown by the peaks at negative chemical shifts that give a characteristic "arrowhead" pattern (labeled in Figure 7). Therefore, a nonpolar side chain that is on an opposite face of the helix from the position of the sequence substitutions is minimally affected. But the amine protons of all four of the Trp indole rings are shifted 0.2−0.3 ppm downfield (Table 4).

Heterodimer Experiments

Heterodimer experiments also confirm the RH helix sense and the structural equivalence of [Ala[1],D-Ala[2]]gA with [Val^1,Gly^2]gA. If the backbone structure of [Ala[1],D-Ala[2]]-gA were dramatically perturbed, hybrid channels would not form (Durkin et al., 1992; Koeppe et al., 1992). As illustrated in Figure 8, hybrid channels form between [Ala[1],D-Ala[2]]gA and [Val^1,Gly^2]gA, as evidenced by the presence of a new channel type when the two gramicidins are present together. This new channel type is seen in the single-channel current trace, as events with a current amplitude that is intermediate to that of the two symmetric channels. Correspondingly, the current transition amplitude histogram now has three major peaks; two of these correspond to the two symmetric channel types, while the third (in the middle) corresponds to the hybrid channels (Figure 8, middle). Hybrid channels form freely between [Val^1,Gly^2]gA and [Ala[1],D-Ala[2]]gA, as the normalized hybrid channel appearance [$f_h/2(f_a f_b)^{0.5}$], where f is the channel appearance rate and

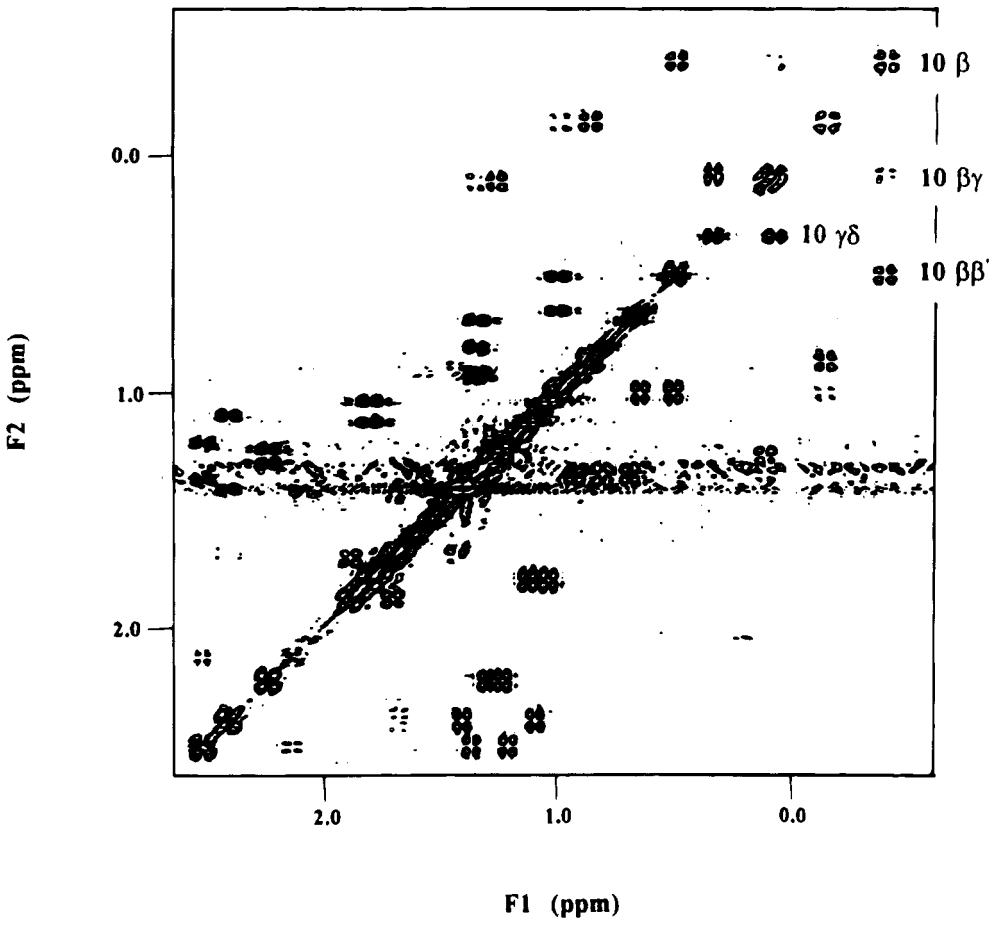

FIGURE 7: High-field region of the DQCOSY spectrum of [Ala¹,D-Ala²]gA in SDS, 55 °C. The spectrum is very similar to the equivalent region of the [Val¹,Gly²]gA spectrum, with the same strong shielding of Leu¹⁰ by an aromatic ring (Koeppe et al., 1995). Selected peaks are labeled with the 10 β being the point of the "arrowhead" (see text).

Table 4: NMR Chemical Shifts (ppm) for the Trp Backbone and Indole Ring N−H Protons of gA and [Ala¹,D-Ala²]gA

	backbone N−H		indole N−H	
	[Ala¹,D-Ala²]gA	gA[a]	[Ala¹,D-Ala²]gA	gA[a]
Trp⁹	9.20	8.98	10.05	9.73
Trp¹¹	8.79	8.73	9.89	9.62
Trp¹³	8.68	8.60	9.94	9.76
Trp¹⁵	8.97	8.81	10.06	9.87

[a] From Arseniev et al. (1985).

the subscripts h, a, and b denote the hybrid and the homodimeric channels, respectively, is 1.01 [see Durkin et al. (1990)]. The average hybrid channel duration is equal to the geometric mean of the two symmetric channels, $\tau_h/(\tau_a\tau_b)^{0.5} = 0.98$, which indicates that there is no strain at the join between the monomers. These results show that the backbone structure of [Ala¹,D-Ala²]gA channels is generally similar and compatible to that of [Val¹,Gly²]gA channels, which is a single-stranded RH $\beta^{6.3}$ helix. This finding is consistent with the structural motif indicated by the [Ala¹,D-Ala²]gA CD spectra in DMPC and SDS and the NMR spectrum in SDS.

Gly² → D-Ala² Substitution in the Native [Val¹,Gly²]gA. To examine the effect of D-Ala² in the context of L-Val¹, [Val¹,D-Ala²]gA was synthesized. The changes in channel properties generally follow the trends seen for the other Gly² → D-Ala² substitutions. The conductance for 1.0 M NaCl at 200 mV is 14.6 pS for [Val¹,Gly²]gA channels and 16.7 pS for [Val¹,D-Ala²]gA channels, a 12% increase (Table 2B; Figure 9). As with the other Gly² → D-Ala² substitutions, there is a decrease in the Cs^+ conductance (Table 3B). With [Val¹,D-Ala²]gA, the conductance decrease is from 47.2 to 40.6 pS (~15%). All analogues with a D-Ala² have a lower Cs^+/Na^+ selectivity.

The average duration for [Val¹,Gly²]gA channels in NaCl is 800 ms and for [Val¹,D-Ala²]gA channels is 3300 ms, a 4-fold increase (Table 2A; Figure 9). For [Val¹,D-Ala²]gA channels the average duration in CsCl is reduced (by ~20%) relative to that in NaCl. The difference is small and within the normally observed variation [cf. Durkin et al. (1993)].

DISCUSSION

In this article we address the question of how residue substitutions at the interface between the monomers in a membrane-spanning channel can alter channel properties. Specifically, we have investigated the systematic replacement of the first two amino acids of [Val¹,Gly²]gA with the two smallest amino acids, Gly and Ala, while retaining the strictly alternating L,D-amino acid sequence found in [Val¹,Gly²]gA. The key result is the stabilizing effect of D-Ala² (as compared to Gly²).

We first address the question of structural equivalence and will conclude that the analogue channels are equivalent to [Val¹,Gly²]gA channels, which is a single-stranded RH formyl-NH-to-formyl-NH $\beta^{6.3}$ helix in lipid bilayer and lipid-like environments. Next the duration and conductance results will be discussed. We conclude that the effects of the

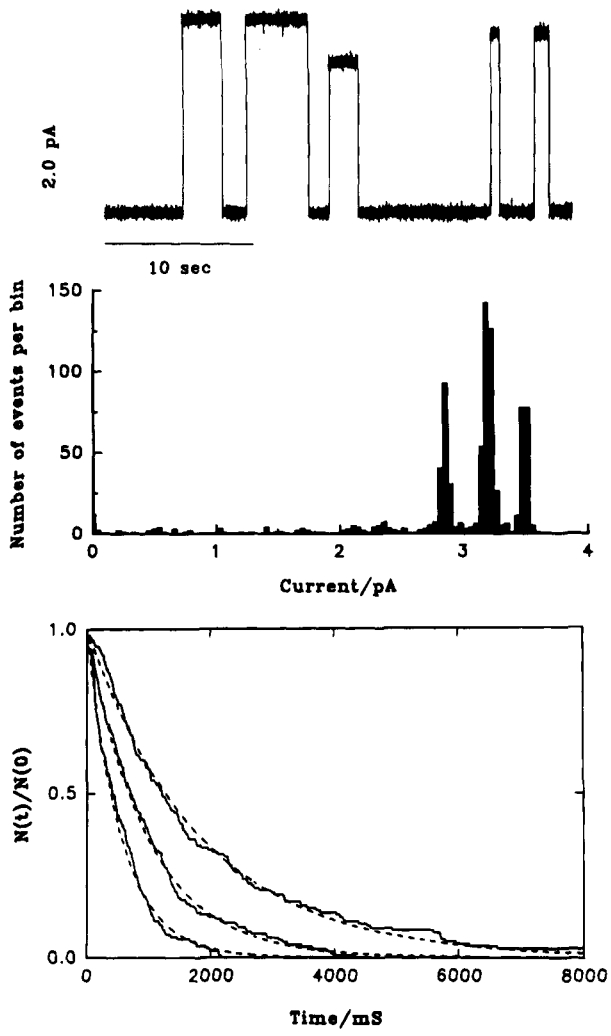

FIGURE 8: Hybrid channel formation between [Ala^1,D-Ala^2]gA and [Val^1,Gly^2]gA. (Top) Single-channel current trace observed after the addition of [Ala^1,D-Ala^2]gA and [Val^1,Gly^2]gA to both sides of the bilayer. There are three channel types: symmetric [Ala^1,D-Ala^2]gA channels (the first two events); symmetric [Val^1,Gly^2]gA channels (the third event); and heterodimeric [Ala^1,D-Ala^2]gA/[Val^1,Gly^2]gA channels (the last two events). (Middle) Current transition amplitude histogram obtained in the presence of [Ala^1,D-Ala^2]gA and [Val^1,Gly^2]gA. There are 829 events in the histogram. The three peaks represent (from left to right) symmetric [Val^1,Gly^2]-gA channels, i = 2.8 pA, 187 events (23%); heterodimeric [Ala^1,D-Ala^2]gA/[Val^1,Gly^2]gA channels, i = 3.2 pA, 363 events (44%); symmetric [Ala^1,D-Ala^2]gA channels, i = 3.5 pA, 172 events (21%). The ratio $f_h/[2(f_a f_b)^{0.5}] = 1.01$ [cf. Durkin et al. (1990)]. (Bottom) Duration distributions for the three channel types. The solid curves denote normalized survivor plots for the channel durations, the interrupted curves denote maximum-likelihood fits of single-exponential distributions $[N(t)/N(0)] = \exp(-t/\tau)$, where $N(t)$ denotes the number of channels with a duration longer than t, and τ is the average duration. [Val^1,Gly^2]gA channels have the shortest duration, τ = 560 ms, the [Ala^1,D-Ala^2]gA channels have the longest duration, τ = 1800 ms; the [Ala^1,D-Ala^2]gA/[Val^1,Gly^2]gA channels have an intermediate duration, τ = 980 ms. The ratio $\tau_h/(\tau_a\tau_b)^{0.5} = 0.98$.

substitutions at positions one and two with respect to the duration and conductance are mainly independent. The average durations decrease when Val^1 is replaced but increase with a Gly^2 → D-Ala^2 substitution. The conductance results show that single substitutions alter the conductance but the substitutions are not additive and may be in different directions with Cs^+ or Na^+ as permeant ions.

Structural Equivalence. [Val^1,Gly^2]gA and its analogues can adopt several different conformations (Bystrov & Arseniev, 1988). The relative distributions among these conformations vary as a function of the environment and the amino acid sequence. Conformations that could possibly be assumed by gramicidin in lipid bilayer membranes include RH or LH single-stranded $\beta^{6.3}$ helices, and double-stranded parallel or antiparallel helices (Urry, 1971; Veatch & Blout, 1974; Bañó et al., 1991; Koeppe et al., 1991; Durkin et al., 1992). The double-helical conformers usually do not function as channels [but see Koeppe et al. (1991) and Durkin et al. (1992)]. By itself, each gramicidin analogue forms only a single predominant conducting channel type (Figures 2 and 9A,B) which indicates that there is only one channel conformation [see also Durkin et al. (1990, 1993) and Koeppe et al. (1992)]. [Val^1,Gly^2]gA and analogues that maintain the strictly alternating L,D sequence normally produce channels that are RH single-stranded $\beta^{6.3}$ helices. Generally, only analogues of gramicidin with reversed chirality form LH channels (Koeppe et al., 1992). An exception is [L-Val^5,D-Ala^8,L-Leu^{9,11,13,15},D-Trp^{10,12,14}]gA (an analogue with nine sequence changes), which can form heterodimers with both [Val^1,Gly^2]gA (RH) and [D-Val^1,Gly^2]-gA$^-$ (an analogue that reverses the chirality of each residue and forms LH channels) (Saberwal et al., 1994).

Three distinct lines of evidence—CD spectra, NMR spectra, and hybrid channel experiments—all show that a change of both residues one and two to Ala does not significantly change the structure of gramicidin in DMPC, SDS, or DPhPC. The conservation of the single-stranded RH $\beta^{6.3}$ gA helical structure is shown by (i) the similarity of the [Ala^1,D-Ala^2]gA CD spectrum to the gA channel CD spectrum, (ii) the NOE intrahelical interactions in the fingerprint region of the 2-D ^{1}H NMR for [Ala^1,D-Ala^2]gA in SDS with 10% TFE [which has been observed for [Val^1,Gly^2]gA by Arseniev et al. (1985) and for [Val^5,D-Ala^8]gA by Koeppe et al. (1994b)], and (iii) the formation of hybrid channels with [Val^1,Gly^2]gA. The CD and NMR spectra of [Ala^1,D-Ala^2]gA suggest that there are slight differences in the backbone and the side chains. In the CD spectrum the ellipticities at 218 and 235 nm are reduced compared to gA, which could reflect subtle changes in the static orientation and/or dynamics of the indole side chains. In the NMR spectrum, changes in the chemical shifts of some of the NH and CαH hydrogens indicate that some backbone changes take place away from the substitutions. More importantly, small changes in the orientations of Trp side chains are indicated by the changes in the NMR spectrum of [Ala^1,D-Ala^2]gA. Given the similar backbone structures, the functional differences between gA and [Ala^1,D-Ala^2]gA channels can be attributed to the altered side chains at positions one and two of the analogue and to the resulting differences in side chain orientations (possibly including altered Trp orientations), but we cannot exclude small modulations of the backbone peptide plane tilt angles within the RH $\beta^{6.3}$ framework.

Channel Durations. Gramicidin channels form when monomers incorporate into the bilayer from the opposing sides (O'Connell et al., 1990) and associate via hydrogen bonds between the first five residues of each monomer. These dimeric gramicidin channels are in equilibrium with non-conducting monomers (Hladky & Haydon, 1972; Bamberg & Läuger, 1973; Veatch & Stryer, 1977; Cifu et al., 1992),

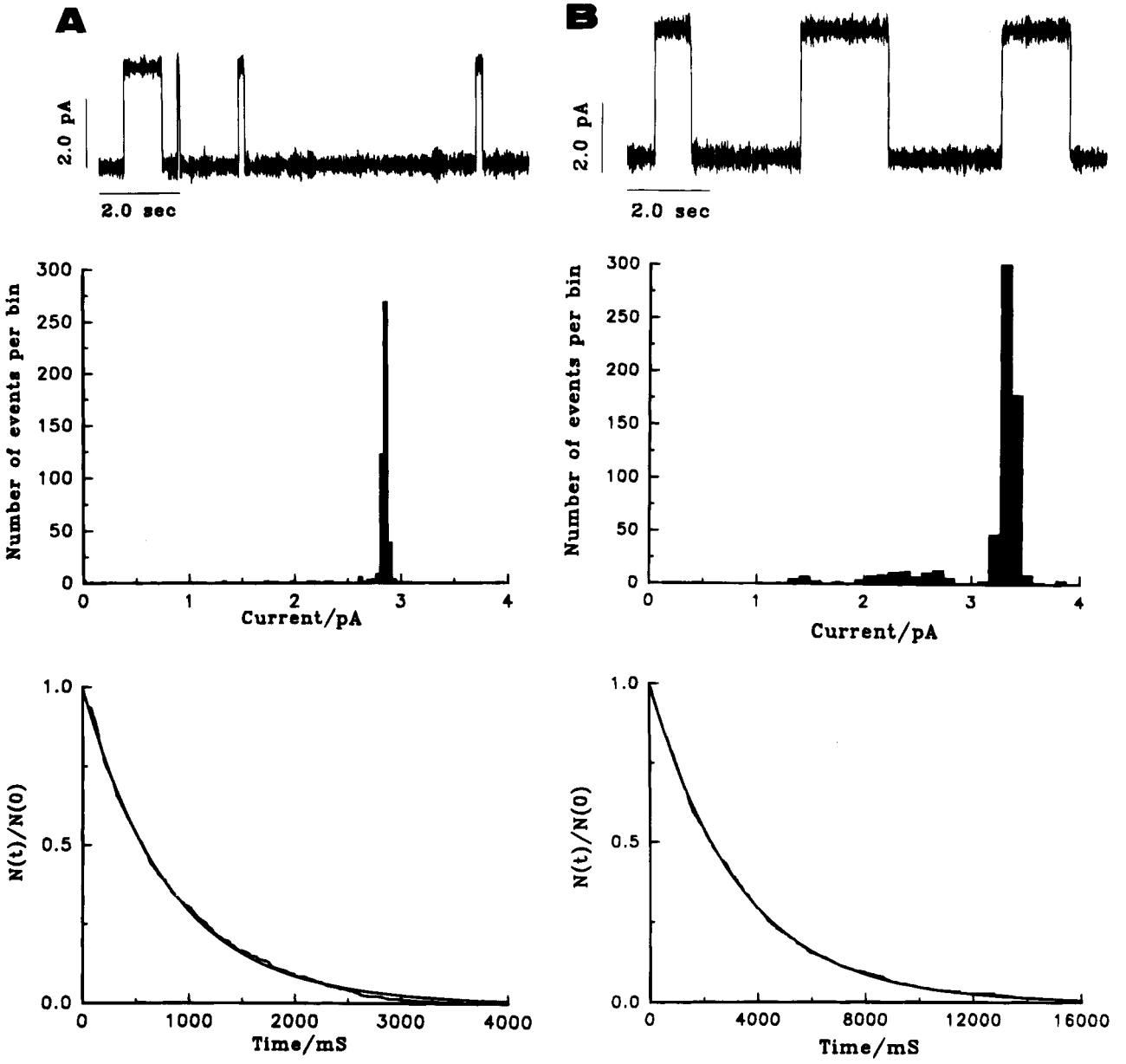

FIGURE 9: Channel formation by [Val[1],Gly[2]]gA and [Val[1],D-Ala[2]]gA. (A) Results for [Val[1],Gly[2]]gA. (B) Results for [Val[1],D-Ala[2]]gA. For each analogue, the figure shows (from top to bottom) a single-channel current trace, current transition histogram, and normalized duration distribution with single-exponential fit. The calibration bars for the current traces denote 2 pA (vertically) and 2 s (horizontally). The average single-channel conductances and durations are listed in Table 2. Conditions: 200 mV, 1.0 M NaCl.

and there is no evidence for nonconducting dimers (Veatch et al., 1975). Furthermore, analysis of the distribution of intervals between conducting events shows evidence for only a single nonconducting state (Andersen, 1978; Oiki et al., 1994). The average duration (τ) of a channel is therefore a measure of the rate of conversion from a conducting dimer to nonconducting monomers. The amino acids at the interface of the two monomers and channel–lipid interactions are two factors that affect this monomer–dimer equilibrium (Andersen & Koeppe, 1992; Lundbæk & Andersen, 1994). It would be expected that monomer–monomer contacts may be altered when any of the first five residues are altered.

Analogues with substitutions at position one show a relationship between the size and shape of the residue and the average duration of the channels formed by the analogues. Replacing the branched Val[1] by a straight aliphatic side chain (e.g., norvaline or norleucine) decreases the channel duration (Russell et al., 1986; Durkin et al., 1990). This result also holds for a Val[1] → Ala[1] substitution which decreases the overall "bulk" at the dimer interface by deleting a branched side chain on each monomer (Tables 2 and 3). This may alter the side chain–backbone interactions and/or the interactions between residues 1 and 5 on different monomers (Figure 10).

A Gly → D-Ala substitution at the second position increases the average channel duration in all cases investigated. Although the analogue [Gly[1],D-Ala[2]]gA has the destabilizing Gly[1], it nevertheless forms channels with a remarkably long average duration (1100 ms), and the duration of [Ala[1],D-Ala[2]]gA channels is again further increased (Table 2). D-Ala[2] is opposite D-Leu[4] (Figure 10), and one is tempted to infer that Ala–Leu interactions may be favorable for channel stability. This is, however, not the case with D-Leu[4]. When D-Leu[4] in [Ala[1],D-Ala[2]]gA is replaced by D-Ala to give [Ala[1],D-Ala[2,4]]gA, the average channel duration in 1 M CsCl is further increased to 6000

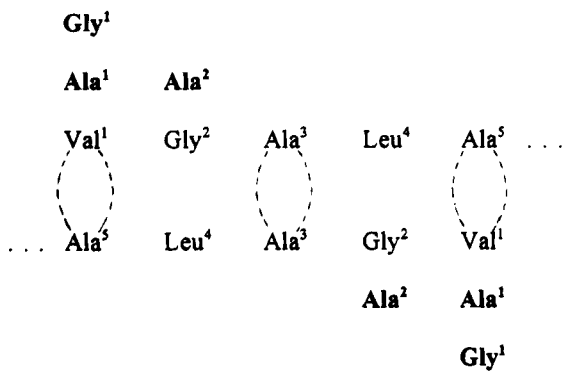

FIGURE 10: First five residues of [Val¹,Gly²]gA and the analogues examined here to show which side chains could be interacting across the dimer junction. The six intermolecular hydrogen bonds linking residues 1...5, 3...3, and 5...1 at the dimer junction are shown schematically.

ms (Koeppe et al., 1994b), as opposed to 2400 ms for [Ala¹,D-Ala²]gA (Table 3). The D-Leu⁴ → D-Ala⁴ replacement thus suggests that local side chain−backbone interactions are important for channel stability. We note the [Ala¹,D-Ala²,⁴]gA has alanines for all of the first five residues.

A second factor that affects channel duration is the channel−membrane interactions. When a gramicidin channel forms, the adjacent membrane will be deformed; there will be an energetic cost associated with the local membrane deformation (Elliot et al., 1983; Huang, 1986; Helfrich & Jakobsson, 1990; Andersen et al., 1992; Lundbæk & Andersen, 1994), and the duration of channels will vary with length of the analogue (Durkin et al., 1993). Because [Val¹,Gly²]-gA and all of the analogues considered here are of equal length, the energetic cost for forming a channel within the lipid bilayer should be approximately the same. The alteration in average duration shows that the particular amino acid substitutions in these cases are important determinants of the channel duration.

The changes in channel durations observed with Na^+ were confirmed in experiments using Cs^+ as the permeant ion (Tables 2 and 3), and the results were similar, except for [Val¹,D-Ala²]gA. The [Val¹,D-Ala²]gA duration with Cs^+ is reduced compared to Na^+. This result goes against the trend in the other experiments reported here; but the variation is within the range of day-to-day variations in average duration for a given channel type. Nevertheless, the Val¹-D-Ala² sequence at the formyl-NH-terminus may constrain backbone movement so much that it, while stabilizing the channel overall, causes a strain at the junction between the monomers when Cs^+ is passing through the pore.

Single-Channel Conductance. Given a fundamentally invariant backbone structure (see above), changes in the magnitude of a channel's conductance are due primarily to subtle modulations of the structure or to interactions between the permeant ions and side chain dipoles, which are affected by the orientations of the side chains (Mazet et al., 1984; Koeppe et al., 1990; Andersen et al., 1992). The sequence substitutions examined here are in the middle of the lipid bilayer where the amino acids can affect the central barrier for translocation, perhaps through subtle modulations of the backbone end structures where the monomers meet each other. Nevertheless, longer-range subtle structural adjustments were also noted in the CD and NMR results (see above). We cannot, at present, evaluate whether single-channel conductance changes should be ascribed primarily to alterations near the point where the sequence was changed or to longer range effects.

Several trends in the conductance in Na^+ are apparent for the analogue channels. (i) [Ala¹,Gly²]gA shows that the elimination of the branching of the Val¹ side chain increases the conductance by ~10%. (ii) A similar conductance increase is achieved by substituting D-Ala for Gly² in [Val¹,Gly²]gA, but the Ala¹ and Ala² substitutions are not additive, as there is no further conductance change on introducing a second Ala in [Ala¹,Gly²]gA. (iii) With Gly¹ there is a 40% decrease in the Na^+ conductance, independent of whether Gly or Ala is at position two. (iv) The conductance is much more sensitive to Gly at position one than to Gly at position two. The basis for these interactions is elusive but could be due to a shift of the orientations of some peptide planes and/or some side chains along the channel. The 2-D ^1H NMR of [Ala¹,D-Ala²]gA shows a shifting of the Trp⁹ amide NH and CαH resonances, which would indicate a slight change in the backbone and thus an effect on the position of the Trp⁹ carbonyl oxygen within the pore of the channel. Also, the orientation of the Trp indole rings (again consistent with the ^1H NMR) could be slightly different than in [Val¹,Gly²]gA, such as to give analogues with Ala in the first and/or second position higher conductances.

Single-channel conductances for Cs^+ were also measured. The pattern varied somewhat from the Na^+ results. In all cases, a Gly or Ala at position one produced channels with Cs^+ conductances less than [Val¹,Gly²]gA channels. As with Na^+, an additional substitution of D-Ala² in [Gly¹,Gly²]gA did not significantly change the Cs^+ conductance. However, the substitution of D-Ala² in [Ala¹,Gly²]gA caused a further decrease in the conductance; in this case, the substitutions are additive. These differences could be due to slight changes in the orientations of the backbone carbonyl oxygens that alter the Na^+/Cs^+ ion selectivity.

Summary. For gramicidin analogues with either Val, Ala, or Gly at position one, a Gly² → D-Ala² substitution increases the average channel duration, with smaller effects on conductance and little effect on channel structure. The stabilization by D-Ala² shows the importance of the precise placement of specific amino acids. More generally, these results form part of an effort to understand the molecular interactions that are involved in channel assembly, stability, and function.

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