Formation of non-$\beta^{6.3}$-helical gramicidin channels between sequence-substituted gramicidin analogues

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ABSTRACT Using the linear gramicidins as an example, we have previously shown how the statistical properties of heterodimeric (hybrid) channels (formed between the parent [Val^1]gramicidin A (gA) and a sequence-altered analogue) can be used to assess whether the analogue forms channels that are structurally equivalent to the parent channels (Durkin, J. T., R. E. Koeppe II, and O. S. Andersen. 1990. *J. Mol. Biol.* 211:221–234). Generally, the gramicidins are tolerant of amino acid sequence alterations. We report here an exception. The optically reversed analogue, gramicidin M$^-$ (gM$^-$) (Heitz, F., G. Spach, and Y. Trudelle. 1982. *Biophys. J.* 40:87–89), forms channels that are the mirror-image of [Val^1]gA channels; gM$^-$ should thus form no hybrid channels with analogues having the same helix sense as [Val^1]gA. Surprisingly, however, gM$^-$ forms hybrid channels with the shortened analogues des-Val^1-[Ala^2]gA and des-Val^1-gC, but these channels differ fundamentally from the parent channels: (*a*) the appearance rate of these heterodimers is only ~1/10 of that predicted from the random assortment of monomers into conducting dimers, indicating the existence of an energy barrier to their formation (e.g., monomer refolding into a new channel-forming conformation); and (*b*), once formed, the hybrid channels are stabilized ~1,000-fold relative to the parent channels. The increased stability suggests a structure that is joined by many hydrogen bonds, such as one of the double-stranded helical dimers shown to be adopted by gramicidins in organic solvents (Veatch, W. R., E. T. Fossel, and E. R. Blout. 1974. *Biochemistry.* 13:5249–5256).

INTRODUCTION

A powerful tool to study structure-function relations in proteins is the use of site-directed mutagenesis to alter the sequence of the protein in question. A general question when using this approach is: do the amino acid sequence alterations alter the structure of the protein? This question becomes especially important in the case of membrane-spanning ion channels. On the one hand, patch-clamp and other single-channel recording methods provide detailed information about the function of single channels (molecules); on the other hand it is difficult to produce (and purify) the large quantities of protein required for biochemical and structural studies. One is thus faced with a discordance between a very precise description of function and a near-absence of structural information.

In any case, mechanistic interpretations of changes in function depend on the assumption that the protein's structure (whether it is known to a high resolution or only surmised from modeling studies) is unchanged by the sequence alteration, except for the alterations in side chain geometry and whatever local contacts may need to be adjusted. This approach is based on the reasonable presumption that, if the qualitative behaviors of the mutant and native protein are the same and the mutation introduces only quantitative differences (e.g., a change in conduction, an increase in open time, a prolongation of inactivation), then the protein must have the same global structure because it still works. If, on the other hand, a given mutation induced a qualitative difference, e.g., if it completely abolished function, that could be the result of a global failure to fold.

We tested this presumption using the gramicidin channels as a prototypical family of membrane-spanning proteins. Gramicidin A (gA) is a fifteen-amino-acid peptide that forms channels that exhibit near-ideal selectivity to small monovalent cations (Hladky and Haydon, 1972; Myers and Haydon, 1972). The sequence of [Val^1]gA is given in Table 1.

Standard gramicidin channels are head-to-head single-stranded dimers of right-handed $\beta^{6.3}$ helices; the evidence is reviewed by Durkin et al. (1990) and Andersen et al. (1992). As originally proposed by Urry (1971), the peptide chain is rolled into a helix having 6.3 residues per turn, with adjacent turns joined by intramolecular hydrogen bonds between the carbonyl and amide groups of the backbone. All side chains are on the exterior of the helix, and the peptide groups form the walls of a pore with a luminal diameter of ~0.4 nm. Two such helices dimerize by intermolecular hydrogen bonding at their formyl-NH-termini to form the functional channel.

Not only are gramicidin channels dimers, but het-

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TABLE 1 Gramicidin analogue amino acid sequences

[Val¹]gramicidin A ([Val¹]gA):

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-$NHCH_2CH_2OH$

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

des-Val¹-gC

HCO---Gly ---------- L-Tyr ----------

des-Val¹-[Ala²]gA

HCO-D-Ala ---------- L-Trp ----------

endo-Gly^{0a}-gC

HCO---Gly-L-Val---Gly---------- L-Tyr ----------

[Val¹]gA^-

HCO-D-Val--- Gly-D-Ala-L-Leu-D-Ala-L-Val-D-Val-L-Val-D-Trp-L-Leu-D-Trp-L-Leu-D-Trp-L-Leu-D-Trp-$NHCH_2CH_2OH$

[Val¹]gM^-

HCO-D-Val---Gly-D-Ala-L-Leu-D-Ala-L-Val-D-Val-L-Val-D-Phe-L-Leu-D-Phe-L-Leu-D-Phe-L-Leu-D-Phe-$NHCH_2CH_2OH$

Nomenclature following IUPAC-IUB (1984).

erodimers can form between a gA molecule and a sequence-modified analogue (e.g., Veatch and Stryer, 1977; Mazet et al., 1984; Durkin et al., 1990). The formation and energetics of functional heterodimers (hybrid channels) demonstrate that the two homodimers have the same (backbone) conformation (Durkin et al., 1990), that is, the channels formed by most sequence-substituted gramicidins are structurally equivalent to [Val¹]gA channels. One can thus show that the linear gramicidins tolerate a large variety of amino acid substitutions at position 1 (Durkin et al., 1990), near the dimer interface, and at positions 9, 11, 13, 15 (Fonseca et al., 1989; Becker et al., 1991), near the channel entrance. Even analogues formed by the insertion or deletion of one residue at the formyl-NH-terminus form channels that are structurally equivalent to gA channels (Durkin et al., 1986; Durkin, submitted for publication).

Here we report an exception. Two gA analogues, the fourteen residue des-Val¹-gramicidin C (des-Val¹-gC) and the optically reversed analogue gramicidin M^- (gM^-) (see Table 1) both form channels that are $\beta^{6.3}$-helical dimers. Des-Val¹-gC channels are right-handed, being structurally equivalent to gA channels (Durkin et al., 1990), while gM^- channels are left handed, being structurally equivalent to the optically reversed gramicidin A (gA^-) channels (Koeppe et al., 1992). Therefore, des-Val¹-gC and gM^- cannot form $\beta^{6.3}$-helical heterodimers (cf Koeppe et al., 1992). Nonetheless, they form hybrid channels, but the behavior of these heterodimers is different from that of either parent channel type, and there is a significant energetic cost associated with the hybrid channel formation. We therefore conclude that these hybrid channels have a fundamentally different conformation than the conventional gramicidin channels.

Some of this material has appeared in preliminary form (Durkin et al., 1987).

MATERIALS AND METHODS

Gramicidin analogues were synthesized by either total synthesis, in the case of gM^- (Heitz et al., 1982) and gA^- (Koeppe et al., 1992), or by semi-synthetic modifications (extensions or deletions) of gramicidin A or C (Durkin et al., 1992). The analogues, along with their sequences, are listed in Table 1 and named according to IUPAC-IUB (1984). The suffixes *A* and *C* refer to the identity of the aromatic residue at position 11 (Trp in gA and Tyr in gC). Because the NH_2-terminal extension is within the formyl moiety, additional residues are named as insertions, with the formyl moiety treated as a virtual residue at position 0.

The experiments were done using the bilayer punch (Andersen, 1983) on planar lipid bilayers formed from diphytanoyl-phosphatidylcholine in *n*-decane (2–3% w/v) at 25 ± 1°C. The electrolyte solution was unbuffered 1.0 M CsCl, which was made up the day of the experiment. The gramicidin analogues were usually added symmetrically to both aqueous solutions bathing the bilayer. In experiments with asymmetrical addition of the gramicidins, the more hydrophobic analogue (gM^-) was always added to the front solution in order to maximize the asymmetrical distribution of the analogues.

The experimental procedures, single-channel current measurements, and analysis were as described in Durkin et al. (1990), with modifications made necessary by the complex behavior of the hybrid channel formed between gM^- and the 14-residue analogues. Des-Val¹-gC/gM^- hybrid channels were mostly characterized from strip-chart records of the results, by counting channel appearances by hand. For this analysis composite channel transitions (see Fig. 8) were counted as a single-appearance/disappearance event. For some experiments the single-channel currents were stored on a digital VCR-based tape-recorder, and the results were analyzed off-line on playback, either computer-assisted or by hand.

The activation energy for heterodimer formation relative to ho-

modimer formation ($\Delta\Delta G^{\ddagger}$) and the standard free energy difference for the heterodimers relative to the homodimers ($\Delta\Delta G^{\circ}$) were estimated as described previously (Durkin et al., 1990). For the present analysis, however, the two heterodimer orientations were not separated:

$$\Delta\Delta G^{\ddagger} = RT \cdot \ln\left[f_h/(2 \cdot (f_a \cdot f_b)^{0.5})\right], \quad (1)$$

and

$$\Delta\Delta G^{\circ} = RT \cdot \ln\left[f_h \cdot \tau_h/(2 \cdot (f_a \cdot \tau_a \cdot f_b \cdot \tau_b)^{0.5})\right], \quad (2)$$

where R and T denote the gas constant and temperature in Kelvin, respectively, whereas the f's and τ's denote channel appearance rates and average durations, respectively; the subscripts denote the two homodimers (a and b) and the heterodimer (h).

The f's were estimated by the number of events (n) in the appropriate peaks in current transition amplitude histograms. The τ's were estimated based on survivor plots of channel lifetimes by fitting a single exponential decay, $N(t) = N(0) \cdot \exp[-t/\tau]$, to the histogram of the number of channels remaining at time t.

RESULTS

A fundamental requirement for structural equivalence among channels formed by different gramicidin analogues is that the chemically dissimilar gramicidins can form heterodimers (hybrid channels) among each other (Durkin et al., 1990). Fig. 1 illustrates results obtained with des-Val^1-gC and endo-Gly^{0a}-gC (Durkin et al., submitted). When either compound is added alone (to both aqueous phases) only a single predominant channel type is seen (upper and middle segments of Fig. 1). These two channel types are the symmetrical homodimers formed by the two gramicidin analogues.

When both gramicidins are added to both aqueous phases, one sees two new channel types. These new channel types are heterodimers, formed by the association of one des-Val^1-gC molecule with one endo-

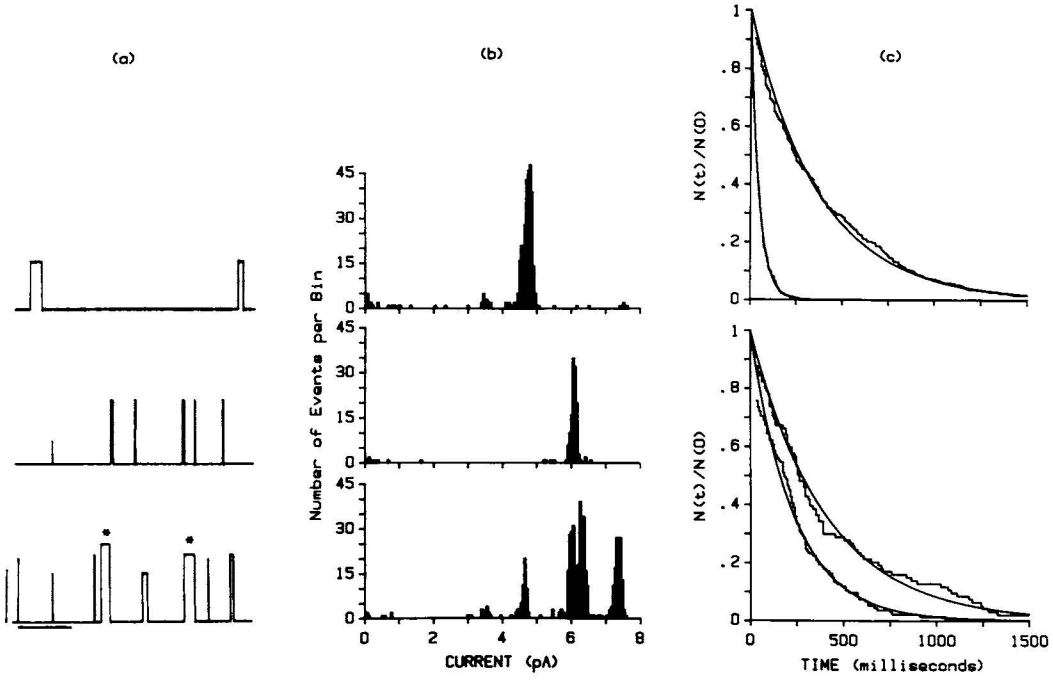

FIGURE 1 Hybrid channel experiment: heterodimers form between des-Val^1-gC and endo-Gly^{0a}-gC. (*a*) Single-channel current traces obtained with only endo-Gly^{0a}-gC (*top*), with only des-Val^1-gC (*middle*), and with their mixture (*bottom*). Both symmetrical channel types appear in the bottom trace as well as two new channel types (denoted by asterisks), the hybrid channels, which only appear when both analogues are present. The calibration bars denote 5 pA (vertically) and 2.5 s (horizontally). (*b*) Current transition amplitude histograms obtained with only endo-Gly^{0a}-gC (*top*), des-Val^1-gC (*middle*), and with their mixture (*bottom*). The top histogram contains 326 transitions, of which 287 (or 88%) are in the main peak at 4.7 pA. The middle histogram contains 139 transitions, of which 124 (or 89%) are in the main peak at 6.1 pA. The bottom histogram contains 498 transitions: 50 (10%) are in the symmetrical channel peak at 4.6 pA; 125 (25%) are in the symmetrical channel peak at 6.0 pA; 146 (29%) are in the hybrid channel peak at 6.3 pA; and 129 (26%) are in the hybrid channel peak at 7.3 pA. (*c*) Survivor histograms for the four channel types observed in these experiments. The top panel shows the results for endo-Gly^{0a}-gC and des-Val^1-gC channels: for des-Val^1-gC channels, τ = 47 ms (N = 160); for endo-Gly^{0a}-gC channels, τ = 360 ms (N = 348). The bottom panel shows the results for the two hybrid channel types: for the high-conductance channels, τ = 230 ms (N = 101); for the low-conductance channels, τ = 400 ms (N = 80).

Gly^{0a}-gC molecule.[1] Both of these gramicidins also form heterodimers with [Val^1]gA, and $\Delta\Delta G^{\ddagger} \simeq 0$ for all three combinations (Durkin et al., submitted). Consequently, des-Val^1-gC and endo-Gly^{0a}-gC channels are both structurally equivalent to [Val^1]gA channels—and of the same handedness.

gM^- forms $\beta^{6.3}$-helical channels that have the opposite handedness of [Val^1]gA (and thus of des-Val^1-gC and endo-Gly^{0a}-gC channels) (Koeppe et al., 1992). Heterodimers should therefore not be able to form between gM^- and either des-Val^1-gC or endo-Gly^{0a}-gC. In accordance with this expectation, when endo-Gly^{0a}-gC and gM^- are added to the same membrane, one observes only the two (symmetrical) homodimeric channels and no hybrid channels (Durkin et al., 1986). But, when des-Val^1-gC and gM^- are added together, a surprising new channel type appears (Fig. 2, *bottom two traces*). As in Fig. 1, these new channels are seen only when both compounds are present; they are therefore hybrid channels. But these hybrid channels have a lower conductance than either symmetrical channel type (cf Figs. 3 and 4) and appear frequently (although the relative appearance rate is not as high as for the des-Val^1-gC/endo-Gly^{0a}-gC heterodimers). As in the case of the des-Val^1-gC/endo-Gly^{0a}-gC heterodimers, there is more than one type of hybrid channel; but it is not clear whether the different hybrid channel types reflect only the different orientations of the channels with respect to the applied potential or a more complicated behavior. The most striking feature of these hybrid channels is, however, their duration (cf Figs. 3 and 4): the average durations of the symmetrical des-Val^1-gC and gM^- channels are 0.04 and 0.34 s, respectively (Fig. 3), while the hybrid channel duration is ~130 s—or 2 min (Fig. 4)! In addition, the hybrid channels exhibit a pronounced "flickery" behavior, with transitions to a low-conductance, or closed, level (see also below).

A similar channel type is seen when des-Val^1-[Ala^2]gA and gM^- are present together in a membrane (Fig. 5). The general features of these hybrid channels are similar to those of the des-Val^1-gC/gM^- hybrids, but their average duration is even longer, ~7 min (cf. Table 2).

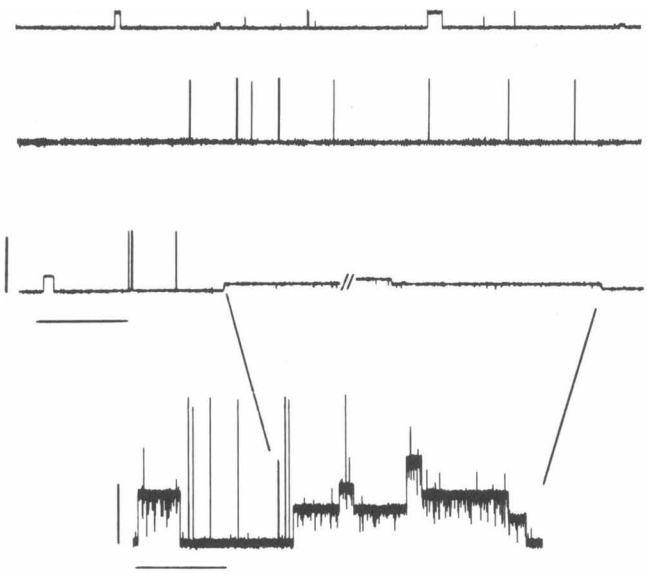

FIGURE 2 Hybrid channel experiment with des-Val^1-gC and gM^-: existence of long-lived hybrid channels. Current traces obtained with only gM^- by itself (*top*), with only des-Val^1-gC (*middle*), and with their mixture (*bottom two traces*). There is a 136 s gap in the upper hybrid channel trace; the lower trace depicts the same segment at a higher amplification and a slower chart speed. Calibration for the upper three traces: 5 pA (vertically); and 5 s (horizontally). Calibration bars for the bottom trace: 1 pA (vertically); and 60 s (horizontally).

Channels formed by gM^- are structurally equivalent to the channels formed by the optically reversed gA^-

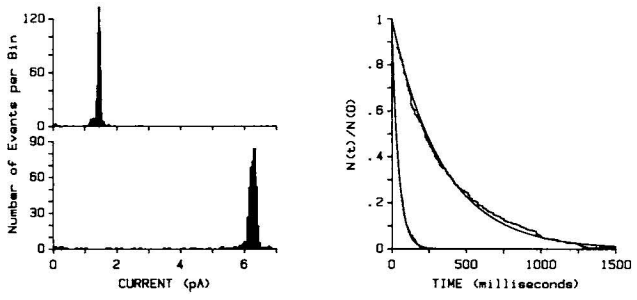

FIGURE 3 Current transition amplitude and single-channel duration histograms for symmetrical gM^- and des-Val^1-gC channels. (*a*) Current transition amplitude histograms obtained with gM^- (*top*), and des-Val^1-gC (*bottom*). The top histogram contains 596 transitions, of which 529 (or 89%) are in the main peak at 1.43 ± 0.05 pA. The bottom histogram contains 439 transitions, of which 395 (or 90%) are in the main peak at 6.2 ± 0.1 pA. (*b*) Survivor histograms for gM^- and for des-Val^1-gC channels: for gM^- channels, $\tau = 340$ ms ($N = 256$); for des-Val^1-gC channels, $\tau = 44$ ms ($N = 518$).

[1]There are two heterodimeric channel types because the join between the monomers is offset from the center of the channel. This results in an asymmetrical free energy profile for ion movement through the hybrid channels, and thus in an asymmetrical current-voltage relation depending on whether the des-Val^1-gC half of the channel faces the positive or the negative solution. Both analogues were added to both aqueous phases, such that both orientations coexist; both branches of the asymmetrical current-voltage relations will therefore appear in the histograms. That the conductances of the heterodimeric channel types are higher than those of the symmetrical channels may likewise result from the hybrid channels' center being offset from the join, because then any local barrier for ion movement across the join (e.g., Jordan, 1987; Pullman, 1987) will be offset from the peak electrostatic barrier for ion movement through the channel.

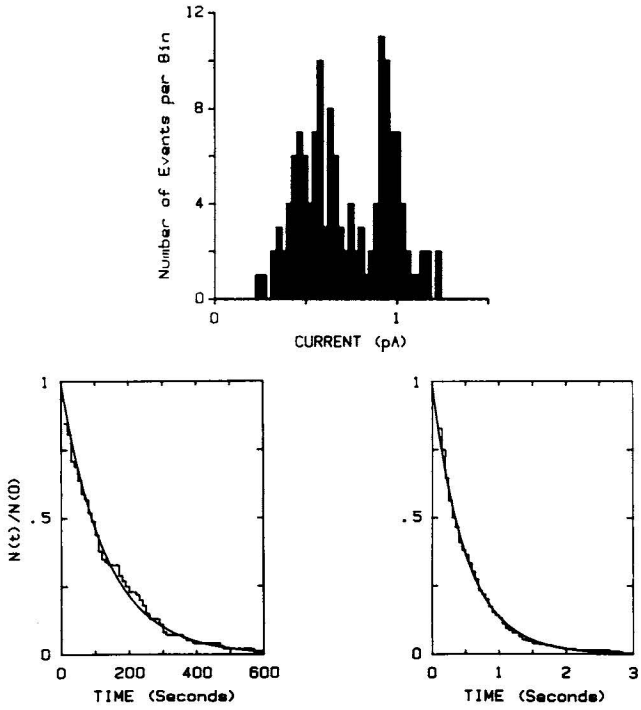

FIGURE 4 Current transition amplitude and duration histograms for des-Val[1]-gC/gM[-] hybrid channels. (*Top*) Amplitude histogram. There are 150 transitions of which 83 are in the broad peak at 0.56 ± 0.13 pA and 47 in the narrow peak at 0.95 ± 0.05 pA. (*Bottom*) Duration histograms. (*Left*) Single-channel durations; composite transitions were counted as single events: τ = 130 s (N = 92). (*Right*) Flicker interval: τ = 480 ms (N = 248). 200 mV, current signal filtered at 200 Hz.

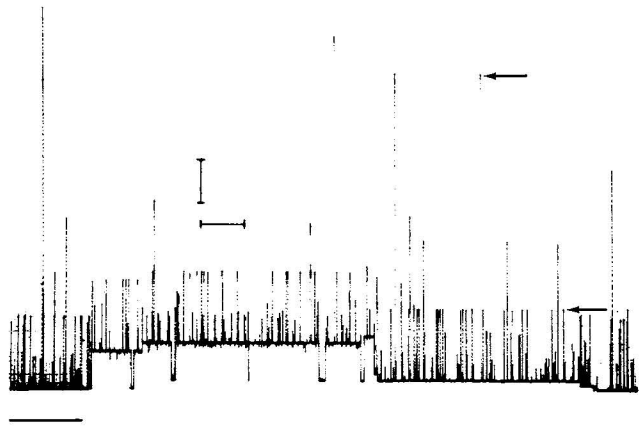

FIGURE 5 Current traces for des-Val[1]-[Ala[2]]gA/gM[-] hybrid channels. The arrows denote the two symmetrical channel types. Calibration bars: 1 pA (vertically); and 60 s (horizontally). 200 mV, current signal filtered at 200 Hz.

TABLE 2 Properties of long-lived gramicidin hybrid channels

(*A*) Single-channel conductances (ps) of symmetrical and hybrid channels

Analogue pair	g_a	g_b	g_h	g_h	g_h
des-Val[1]-gC/gM[-]	30.8 ± 0.8	7.3 ± 0.2	4.2 ± 0.1	2.3 ± 0.4	
des-Val[1]-[Ala[2]]gA/gM[-]	36.0 ± 1.0	7.3 ± 0.2	4.2 ± 0.2	2.7 ± 0.4	0.6 ± 0.3

(*B*) Single-channel durations and relative heterodimer appearance rate

Analogue pair	τ_1	τ_2	τ_h	$n_h/(2(n_a n_b)^{0.5})$
des-Val[1]-gC/gM[-]	0.04	0.3	140	0.1
des-Val[1]-[Ala[2]]gA/gM[-]	0.9	0.2	400	0.1

(*C*) Relative free energies of heterodimer formation and stability

Analogue pair	$\Delta\Delta G‡$ (kJ/mol)	$\Delta\Delta G°$ (kJ/mol)
des-Val[1]-gC/gM[-]	6	−12
des-Val[1]-[Ala[2]]gA/gM[-]	6	−11

(Koeppe et al., 1992). If the hybrid channels formed between des-Val[1]-gC (or des-Val[1]-[Ala[2]]gA) and gM[-] were "simple" $\beta^{6.3}$-helical dimers, one would thus expect that similar hybrid channels would form between des-Val[1]-gC and gA[-], but that is not the case. In fact, no hybrid channels are observed when des-Val[1]-gC and gA[-] are added together (Fig. 6). The formation of long-lived hybrid channels is a particular propensity of gM[-] (in combination with 14-residue gramicidin analogues).

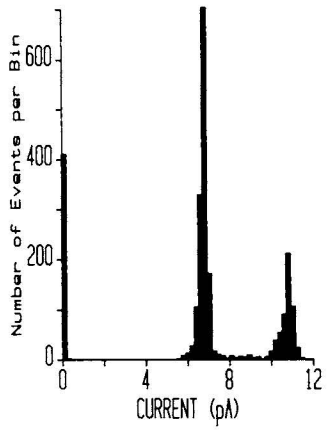

FIGURE 6 Hybrid channel experiment: heterodimers do not form between des-Val[1]-gC and gA[-]. Current transition amplitude histograms obtained when both des-Val[1]-gC and gA[-] are added to aqueous solutions. Channels lasting longer than 12.5 ms were used to determine the current transition amplitudes. The histogram contains 2,386 transitions of which 413 are at the origin (because the discrimination level for channel detection was set very close to the background current noise amplitude). Of the remaining 1,973 transitions, 1,393 (71%) are in the des-Val[1]-gC peak at 6.6 pA, and 543 (28%) are in the gA[-] peak at 10.6 pA.

Hybrid channel formation

Standard (single-stranded) gramicidin channels form by the transmembrane association of monomers (O'Connell et al., 1990). To determine whether Des-Val^1-gC/gM$^-$ hybrid channels form by the same mechanism, des-Val^1-gC and gM$^-$ were added asymmetrically: in one series of experiments both analogues were added to one aqueous solution and none to the other; in the other series, one analogue was added to one solution only, while the other analogue was added to the other solution only. The hybrid channels were seen early in the experiments only when both analogues were added to the same side of the bilayer (Fig. 7). This finding demonstrates that des-Val^1-gC/gM$^-$ hybrid channels form by the association of the two monomers in one half of the membrane.

Hybrid channel behavior

In addition to their long durations, the des-Val^1-gC/gM$^-$ (and des-Val^1-[Ala^2]gA/gM$^-$) hybrid channels also differ from the standard gramicidin channels by virtue of their general behavior. In DPhPC/*n*-decane bilayers, [Val^1]gA channels usually appear as "clean" current transitions, with no intermediate steps being visible at the time resolution of these experiments (cutoff frequency ≤ 1 kHz). Once formed, the channels tend to remain at a stable well-defined conductance level until they disap-

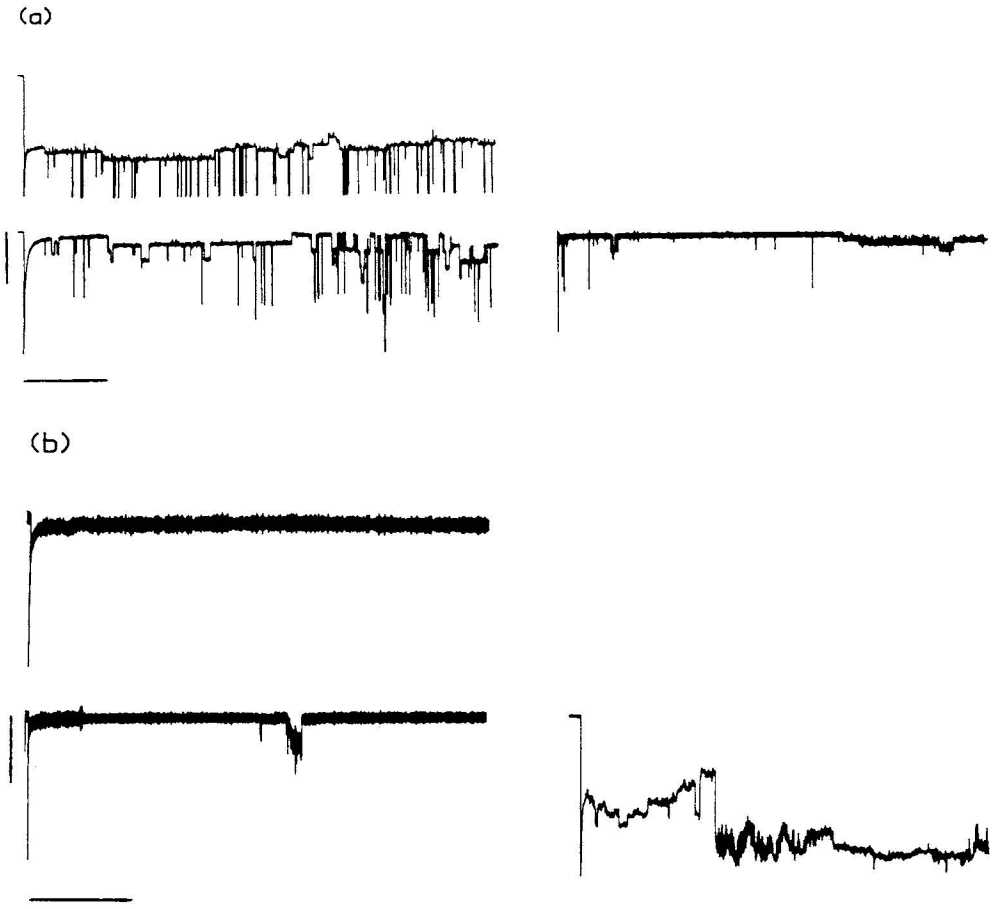

FIGURE 7 Formation of long-lived des-Val^1-gC/gM$^-$ hybrid channels. (*a*) Current traces from two (of four) experiments in which both analogues were added to the same side of a preformed bilayer. Note the presence of both symmetrical and hybrid channels early in all experiments. The trace to the right represents channel activity after the large membrane had been broken and reformed to have both gramicidins present at both interfaces. (*Calibration bars*) vertically, 5 pA; horizontally, 12 s (*left two traces*) and 1 min (*right trace*). (*b*) Current traces from two experiments in which the analogues were added to the opposite sides of a preformed bilayer. Note the low channel activity and the absence of defined channel activity. (Later in the experiment there were a few gM$^-$ channels.) The trace to the right denotes channel activity after the membrane was broken and the gramicidins were allowed to mix. Note the very high channel activity. Calibration bars: vertically, 5 pA; horizontally, 1 min (*left two traces*) and 5 s (*right trace*).

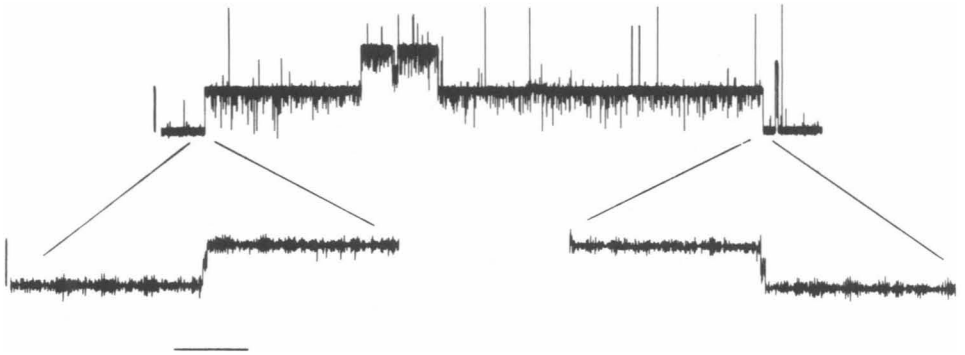

FIGURE 8 Composite appearance and disappearance events of des-Val[1]-gC/gM[-] hybrid channels. The upper trace (filtered at 40 Hz) shows two channels, and a transition to a subconductance state. The lower traces (filtered at 100 Hz) show the composite appearance and disappearance steps. (*Calibration bars*) 1 pA (vertically); and 60 s, upper trace or 1 s, lower trace (horizontally).

pear (cf. Andersen, 1983; Sawyer et al., 1989). Occasional "flickers" or more stable transitions to low-conductance levels are observed, but they are rare (cf Busath and Szabo, 1988; Sawyer et al., 1989).[2] Most gramicidin analogues (and hybrid channels) are similarly well behaved, although a few exhibit rapid transitions between two or more conductance states (Oiki et al., 1992; Durkin et al., submitted for publication).

By contrast, des-Val[1]-gC/gM[-] hybrid channels flicker, have at least two stable conductance levels (Figs. 2 and 4), and frequently appear or disappear through intermediate conductance levels (Fig. 8). The channels can remain at an intermediate level for many seconds before opening fully or disappearing (Figs. 2 and 8), and the brief flickers to the low-conductance state can occur from either an intermediate or the full conductance level. The distribution of intervals between the "flickers" is monoexponential, with an average interval of ~0.5 s (Fig. 4).

Temperature dependence of single-channel durations

Standard gramicidin channels form by the transmembrane association of ($\beta^{6.3}$-helical) monomers coming from opposite monolayers of the bilayer (O'Connell et al., 1990). Channel disappearances thus involve the dissociation of the intermolecular hydrogen bonds at the channel join. In accordance with this picture, the activation energy for [Val[1]]gA channel dissociation is ~80 kJ/mol (Hladky and Haydon, 1972; Bamberg and Läuger, 1974)—which is consistent with the breaking of ~7 hydrogen bonds of ~10 kJ/mol each.[3]

The extremely long durations of the hybrid channels suggest that these channels are stabilized by more than the usual six hydrogen bonds, in which case the temperature dependence of the average duration could be larger than for the standard channels. That was not the case, however: between 15 and 35°C the temperature dependence of the duration of the hybrid channels is comparable to that of the symmetrical homodimers (Table 3), all three channel types having an activation energy of ~130 kJ/mol. The transition states for the disappearance of the symmetrical and the hybrid channels are thus of comparable energy.

Ion selectivity

Des-Val[1]-gC/gM[-] hybrid channels are as cation selective as other gramicidin channels. This was determined in single-channel experiments with asymmetrical CsCl solutions (1.0 M vs. 0.1 M). For technical reasons, these experiments were done at 30°C, where the hybrid

TABLE 3 **Temperature dependence of channel durations**

	τ (s)			E_A (kJ/mol)
	15 °C	25 °C	35 °C	
des-Val[1]-gC	0.2	0.02	0.006	130
gM[-]	2.0	0.2	0.08	120
Hybrids	800	100	20	140

[2]At higher frequencies [Val[1]]gA channels exhibit brief (μs) "flickers" to a closed or low-conductance level, and form and disappear through an intermediate conductance level (Sigworth and Shenkel, 1988; Heinemann and Sigworth, 1990).

[3]The join between the two $\beta^{6.3}$-helical monomers is stabilized by six C=O···H—N hydrogen bonds between the peptide groups and one hydrogen bond from the single-filing water strand in the pore.

channels' conductance is larger (and their average duration shorter). Under these conditions, the reversal potentials (E_{rev}) for the two symmetrical channel types as well as for the full- and intermediate-conductance states of the hybrid channels could be determined individually. They are identical within experimental error (Fig. 9).

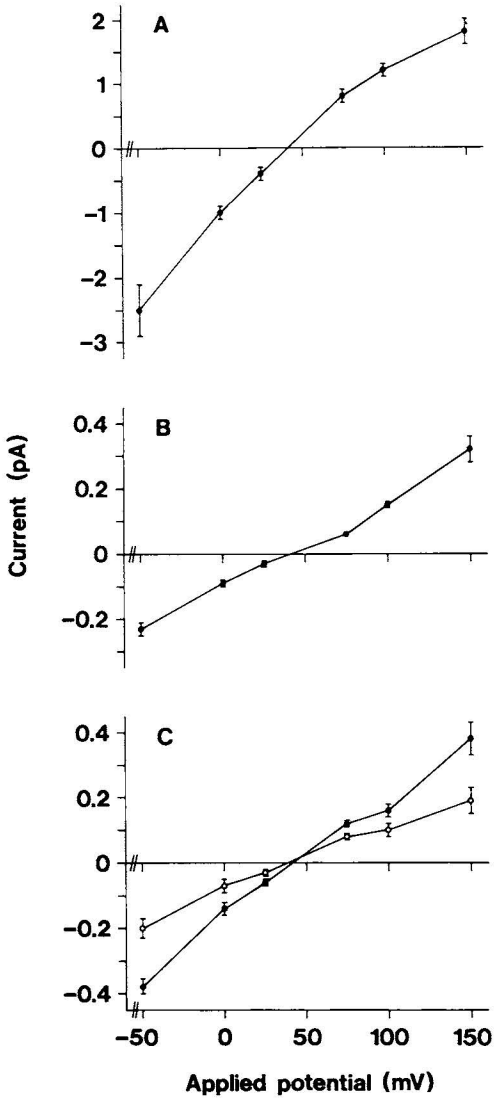

FIGURE 9 Ion selectivity of gramicidin channels. Single-channel current-voltage curves in asymmetrical CsCl solutions. The *cis* chamber contained 0.1 M CsCl, while the *trans* chamber contained 1.0 M CsCl. The applied potential is referenced to the *trans* chamber. (*A*) Results for symmetrical des-Val^1-gC channels ($E_{rev} \simeq +42$ mV). (*B*) Results for symmetrical gM$^-$ channels ($E_{rev} \simeq +42$ mV). (*C*) Results for des-Val^1-gC/gM$^-$ hybrid channels. Closed circles refer to high-conductance events; open circles refer to low-conductance events. $E_{rev} \simeq +42$ mV for both types of events. $T = 30°C$ to decrease the average channel duration (and increase the magnitude of the single-channel current transitions).

The magnitude of E_{rev}, 42 mV, is less than that predicted for an ideally selective channel, 52 mV. This difference is, however, primarily the result of two effects that both act to decrease the magnitude of E_{rev}: (*a*) water flow across the membrane, which will tend to decrease the interfacial ion concentrations (Levitt et al., 1978), an effect which is particularly pronounced within the punch pipette with its large unstirred layer; and (*b*) streaming potentials due to single-file flux coupling between water and ion movement within the channel (Levitt et al., 1978; Rosenberg and Finkelstein, 1978). The important point is that the measured E_{rev} is the same for the symmetrical and hybrid channel types, that is, these hybrid channels are as cation-selective as des-Val^1-gC and gM$^-$ channels.

DISCUSSION

The new channel events reported here are hybrid gramicidin channels because they appear only in the presence of gM$^-$ and a 14-residue analogue. But, unlike the hybrid channels we have examined previously (Durkin et al., 1990; Sawyer et al., 1990; Becker et al., 1991; Durkin et al., submitted for publication) these channels cannot be $\beta^{6.3}$-helical dimers. They must have a fundamentally different conformation.

First, the new hybrid channel appearances are very different in character from standard gramicidin channels. The new channels have stable subconductance states and they "flicker" to closed states. Most striking of all is the enormous prolongation of the durations of these channels: their average duration is three orders of magnitude longer than those of the corresponding symmetrical channels! This should be contrasted with heterodimers formed by gramicidins with position 1 substitutions, where the average durations are intermediate to, or less than, those of the symmetrical channels (Durkin et al., 1990; submitted for publication; Sawyer et al., 1990; Becker et al., 1991). A priori it is difficult to imagine how a hybrid channel with fundamentally the same structure could exhibit such fundamentally different behavior.

Second, no such hybrids are observed between gM$^-$ and 16-residue analogues, such as endo-Gly^{0a}-gC (Durkin et al., 1986; J. T. Durkin and O. S. Andersen, unpublished results). This is in spite of the fact that 14- and 16-residue gramicidin analogues are structurally equivalent (Durkin et al., submitted for publication). Structural equivalence should be a transitive property among channel-formers. Thus, if the long-lived hybrid channels were $\beta^{6.3}$-helical dimers, they should form as

readily between gM^- and 16-residue analogues as between gM^- and 14-residue analogues, but they do not.

Third, the peptide backbone of gM^- channels is stereochemically reversed from that of [Val^1]gA channels (Koeppe et al., 1992), and thus from that of des-Val^1-gC and des-Val^1-[Ala^2]gA channels (Durkin et al., submitted for publication). Consequently, if the des-Val^1-gC/gM^- hybrid channels were $\beta^{6.3}$-helical dimers, it would require that one of the analogues could reverse its helix sense. That analogue would be des-Val^1-gC because hybrid channels were seen with des-Val^1-gC and gM^- but not with endo-Gly^{0a}-gC and gM^-.

But, if des-Val^1-gC could fold as both right- and left-handed β-helices, it would also form both right- and left-handed $\beta^{6.3}$-helical channels (which would show up as two different symmetrical channel types in the current traces and histograms, or as hybrid channels with both right- and left-handed reference compounds). If this were the case, the des-Val^1-gC/gM^- hybrid channel appearance rate would reflect the distribution between left- and right-handed des-Val^1-gC $\beta^{6.3}$-helical monomers. Based on the relative appearance rates of the des-Val^1-gC/gM^- hybrid channels ($\sim$0.1, cf Table 2), however, the ratio of left- to right-handed des-Val^1-gC channels would be only $\sim 1/100$ (see Appendix). Such a small fraction of left-handed des-Val^1-gC channels would not be clearly observable in current traces, amplitude histograms, or duration histograms (Figs. 1–3). By a similar argument, however, the relative appearance rate of des-Val^1-gC/[Val^1]gA^- hybrid channels should be $\sim$0.1, which would be readily detectable. No such substantial fraction of hybrid channels form between des-Val^1-gC and [Val^1]gA^- (Fig. 6).

The absence of hybrid channel formation between des-Val^1-gC and gA^- is particularly important because gM^- forms hybrid channels with either compound. Again, the transitive chain is broken. These results thus rule out the possibility that the des-Val^1-gC/gM^- hybrid channels are (left-handed) β-helical dimers.

Fourth, the long-lived hybrid channels form by a different mechanism than standard gramicidin channels (cf. O'Connell et al., 1990). The results of the asymmetrical addition experiments (Fig. 7) show that a long-lived hybrid channel forms when the two different monomers associate in one half of the bilayer and then proceed to form the membrane-spanning channel. This mechanism is in contrast to the transmembrane association of monomers, from opposite monolayers, to form $\beta^{6.3}$-helical channels.

Finally, the energetics of hybrid channel formation provide possibly the strongest evidence for a fundamentally different conformation of these long-lived hybrid channels. $\Delta\Delta G°$, the standard free energy difference between the hybrid channels and the corresponding symmetrical channels, and $\Delta\Delta G^‡$, the corresponding difference in activation energy, were estimated as described in Methods. The results are summarized in Table 2. In contrast to the situation for analogues with position 1 substitutions (Durkin et al., 1990), where $\Delta\Delta G^‡$ and $\Delta\Delta G°$ are within $RT/2$ of zero, or by single-residue additions or deletions at the formyl-NH_2-terminus (Durkin et al., submitted for publication), where $\Delta\Delta G^‡$ again is within $RT/2$ of zero, but $\Delta\Delta G°$ is positive ($\sim 4 \cdot RT$), we now find that $\Delta\Delta G^‡$ is positive ($\sim 2.5 \cdot RT$) for both analogue pairs, whereas $\Delta\Delta G°$ is negative ($\simeq -5 \cdot RT$). (At 25°C, RT = 2.5 kJ/mol.) The former result implies that the monomers need to refold to form the long-lived hybrid channels (see Durkin et al., 1990 for a detailed discussion). The latter result implies that the overall equilibrium for channel formation is strongly biased in favor of the hybrid channels.

These contrasting changes in $\Delta\Delta G^‡$ and $\Delta\Delta G°$ are easiest to understand if the hybrid channels represent a fundamentally new channel conformation, with the consequent refolding of the analogues constituting an energy barrier.

Possible conformation of the long-lived hybrid channels

If the long-lived hybrid channels are not $\beta^{6.3}$-helical dimers, what, then, are they? Single-channel measurements do not help to answer this question directly. Based on what is known about the structural polymorphism of [Val^1]gA (Veatch et al., 1974; Bystrov and Arsen'ev, 1988; Langs, 1988; Wallace and Ravikumar, 1988), however, the following proposal seems plausible: they may be intertwined (double-stranded) dimers (cf Fig. 10). Double-stranded dimers of an appropriate pitch have the essential features of the standard $\beta^{6.3}$-helical dimer: a pore, lined by the polar peptide groups, which is large enough to accommodate a single-filing

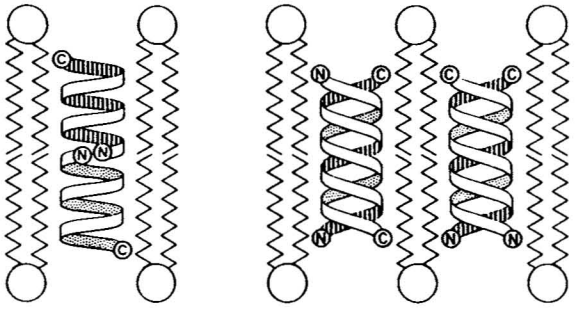

FIGURE 10 Schematic illustration of a single-stranded ($\beta^{6.3}$-helical), right-handed dimer (*left*) as well as two double-stranded (parallel and antiparallel), left-handed dimers (*right*). Modified after Weinstein et al. (1979).

column of water and ions. Double-stranded dimers are joined by 26 C═O···H—N hydrogen bonds (Langs, 1988; Wallace and Ravikumar, 1988), while $\beta^{6.3}$-helical dimers are joined by only six. A larger number of intermolecular hydrogen bonds would explain the stability of the hybrid channels relative to the symmetrical channels, and the presence of several (stable) conductance levels could simply reflect that the monomers could shift relative to one another (by one L-D unit) and still form a membrane-spanning channel. (Indeed, the results of infrared spectroscopy experiments are consistent with the notion that the long-lived des-Val^1-gC/gM$^-$ hybrid channels are double-stranded dimers [T. Earnest and J. T. Durkin, unpublished results].)

If the long-lived hybrid channels are double-stranded dimers, with 26 intramolecular hydrogen bonds, why is the activation energy for their dissociation not much larger than that of standard gramicidin channels (cf. Table 3)? Recall that the long-lived hybrid channels form by a completely different mechanism from that of the standard channels, namely by monomer association in one half of the bilayer (Fig. 7). Although not proven, it is most likely that the heterodimer forms at one membrane-solution interface, and only then can penetrate the membrane to form the membrane-spanning channel. From the principle of microscopic reversibility (Onsager, 1931), the kinetic path of channel disappearances must then retrace the path of channel appearances. Consequently, the first step in a channel disappearance is probably that the channel "loses its grip" in the *trans* membrane-solution interface. At each interface, a channel is expected to form some 6–10 hydrogen bonds to water and to the phospholipid backbone. It is merely fortuitous that the energetics of breaking these contacts are comparable to those of breaking the 6–7 hydrogen bonds at the join of a single-stranded ($\beta^{6.3}$-helical) dimer!

One might ask, why does [Val^1]gA not form similar double-stranded channels, particularly since the double-stranded dimers are the predominant structures in organic solvents (Veatch et al., 1974; Baño et al., 1989)? In fact, [Val^1]gA may do so, but at a very low appearance rate. In experiments with [Val^1]gA, we occasionally observe a characteristic channel type with a conductance ~¼ that of the standard channels and with a duration of tens of seconds. These events comprise on the order of 10^{-4} of the channels, which effectively precludes detailed study and we cannot exclude that they represent some chemical impurity.[4] Nevertheless, it is attractive to speculate that these long-lived events may be double-stranded dimers formed by two [Val^1]gA molecules. (Double-stranded channels have also been proposed to explain the stimulation of cation transport in rat liver mitochondria by desformyl-gA and des(formyl-Val^1)gA [Rottenberg and Koeppe, 1989].)

Generally, however, the formation of the long-lived hybrids is a characteristic of gM$^-$, because similar channels do not form between des-Val^1-gC and gA$^-$ (Fig. 6). As gA$^-$ and gM$^-$ form channels that are structurally equivalent (Koeppe et al., 1992), the molecular basis for the formation of long-lived hybrid channels must reside in the sequence differences between gA$^-$ and gM$^-$, i.e., in the four Trp→Phe replacements in the COOH-terminal half of the molecule. From the point of view of a membrane-spanning molecule, the major difference between a Phe and a Trp is that the indole NH moiety is a hydrogen bond donor (e.g., Stryer, 1988; see also Jacobs and White, 1989; O'Connell et al., 1990; Becker et al., 1991). It is thus likely that the absence of amphipathic Trp residues in gM$^-$ permits (membrane-spanning) folding patterns that are effectively precluded in the case of gA$^-$. For example, at least three (and possibly all four, cf. Becker et al., 1991) of the indole NH's of each monomer of a single-stranded ($\beta^{6.3}$-helical) dimer should be able to hydrogen bond to water or to the phospholipid backbone, while at most two Trps in each monomer of a double-stranded dimer would be able to do so. Membrane-spanning double-stranded des-Val^1-gC/gA$^-$ heterodimers thus will be destabilized, relative to double-stranded des-Val^1-gC/gM$^-$ heterodimers, by the energetic cost of burying one or more indole groups in the membrane interior (by ~10 kJ/mol per indole group) (cf. Schulz and Schirmer, 1979).

The importance of the Trps in the COOH-terminal half of the molecule is further demonstrated by the finding that an analogue of [Val^1]gA with an altered Leu-Trp sequence can form both $\beta^{6.3}$-helical and double-stranded dimeric channels (Koeppe et al., 1991).

Ion permeability

The long-lived des-Val^1-gC/gM$^-$ hybrid channels have conductances that are less than even that of gM$^-$ channels. The low conductance of the hybrid channels is most surprising because all other Phe→Trp substitutions into a gramicidin channel are associated with a conductance increase (Mazet et al., 1984; Becker et al., 1991). A priori, one would thus expect that the hybrid channels (which have more Trp residues) would have a higher conductance than gM$^-$ channels. That expectation is, however, based on results obtained with $\beta^{6.3}$-helical channels, and there is no reason for the generali-

[4]Rosenberg and Finkelstein (1978) reported very long-lived gramicidin channels in bilayers formed from a 1:4 mixture of egg lecithin and cholesterol in *n*-decane. Again, the molecular basis for these events is obscure, but the long-lived events could be double-stranded dimers.

zation to hold true for channels having a fundamentally different structure.

The cation/anion selectivity for the hybrid channels is similar to that of the two symmetrical channels, because E_{rev} is indistinguishable among the different channel types. At this level of description, an important quantifier of channel function is thus preserved when going from the symmetrical to the long-lived hybrid channels, even though the channel structure has changed.

Wallace and Ravikumar (1988) reported the structure of an antiparallel, double-stranded gramicidin dimer: CsCl complex crystallized from methanol, in which each dimer lumen (pore) in the crystal holds three Cl^- ions. This observation raised the possibility that double-helical channels could have significant anion permeability, which is not observed. This apparent discrepancy could arise because the long-lived channels reported here are not, in fact, the antiparallel double-stranded dimer type described by Wallace and Ravikumar (1988). But it could also arise because the very tight packing density in the crystals constrains the positioning of the Cl^- ions sufficiently to "force" them into the pore, whereas in an isolated channel electroneutrality would be preserved by keeping the Cl^- in the aqueous solutions (Wallace and Ravikumar, 1988).

Implications for other systems

The results in this article and in our previous work on gramicidin hybrid channels (Durkin et al., 1990; submitted for publication) show that one is faced with an energetic window with respect to useful perturbations of a channel's structure: if a mutant channel is stabilized or destabilized by more than ~20 kJ/mol, the resulting construct is nearly impossible to observe. In our previous work, heterodimers between [Val^1]gA and des-Val^1-gC were destabilized by ~10 kJ/mol, and this destabilization was manifested in the hybrid channels' average duration being ~4 ms. If the hybrid channels were destabilized by another 10 kJ/mol, their average duration would have been 60 μs, which in conjunction with their small conductance would have made them virtually unobservable. Conversely, in this work an additional 10 kJ/mol stabilization of the des-Val^1-gC/gM$^-$ hybrids would have resulted in an average open time of 7 h! Such channels would be indistinguishable from leaks (and would make us miss dinner to boot). An important point of reference in this respect is that 10 kJ/mol corresponds approximately to the making or breaking of one hydrogen bond (cf. Schulz and Schirmer, 1979).

A similar energetic window will likewise limit the detection of mutant channels expressed in heterologous systems. Channels with very short open times may not be detectable. Channels with very long open times may appear only as leaks and thus compromise cell volume control, which may be fatal to cells that express the mutant channel.

Lastly, we have shown how seemingly modest sequence alterations can profoundly alter the structure of a membrane-spanning channel. This result may be an extreme example of the problems that one might face, but it raises the question, what does it mean that the structure is conserved? In any protein, if the primary structure has been altered, the structure must be somewhat altered. But is it important? Fundamentally, the question, whether the structure is conserved, boils down to the question, whether a functional alteration induced by a sequence substitution can be interpreted as a local perturbation, i.e., whether it can be understood solely on the basis of the different physico-chemical characteristics of the native and the substituted amino acid side chains in the vicinity of the substitution, or whether more global changes are involved (e.g., Hibler et al., 1987; Alexandrescu et al., 1990). It is in this respect also important to note that either of the gramicidins by itself forms single-stranded ($\beta^{6.3}$-helical) dimers. It is only when combined that they form the new channel type.

Conclusions

Two gramicidin analogues that by themselves form standard ($\beta^{6.3}$-helical) channels can, when combined, form an assembly with a radically different conformation. This shows that the preferred conformation of a molecule may depend on its partner in an oligomeric construct. The real surprise, however, is that this new conformation is itself a functional channel. Preservation of function does not necessarily imply the preservation of structure.

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APPENDIX

How many left-handed des-Val^1-gC channels should be observed if the long-lived hybrids were left-handed $\beta^{6.3}$-helical dimers?

We assume that left-handed des-Val^1-gC and gM$^-$ $\beta^{6.3}$-helices form heterodimers that are structurally equivalent, which means that $\Delta\Delta G^{\ddagger} = 0$ when evaluated relative to the left-handed des-Val^1-gC channels. Letting f_h, f_a and f_b' denote the appearance rates for the long-lived hybrids, symmetrical gM$^-$ channels and left-handed des-Val^1-gC channels, respectively, we thus have, cf. Eq. 1, that

$$f_h/(2 \cdot (f_a \cdot f_b')^{0.5}) = 1. \quad \text{(A1)}$$

Relative to the right-handed des-Val[1]-gC channels, however, we have that

$$f_h/(2 \cdot (f_a \cdot f_b'')^{0.5}) = r, \quad \text{(A2)}$$

where f_b'' denotes the appearance rate for the right-handed channels (the events in the major peak in the current amplitude histogram), and r denotes relative hybrid channel appearance rate. Combining Eqs. A1 and A2,

$$f_b'/f_b'' = r^2. \quad \text{(A3)}$$

In the case of des-Val[1]-gC/gM[-] hybrids, $r \simeq 0.1$ (Table 2), and the distribution between left- and right-handed membrane-spanning $\beta^{6.3}$-helical dimers should be $\sim 1/100$.

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DISCUSSION

Session Chairman: Ronald Kaback *Scribe:* Murray Becker

DAVID BUSATH: You propose that the long-lived heterodimers are intertwined-helices. However, can you experimentally distinguish between the intertwined helical dimer and higher order multimers such as 3- or 4-stranded barrels?

JOHN DURKIN: I do not believe that we have a practical technique for distinguishing between dimers and higher order aggregates. In principle, we could answer this question by observing the heterodimer formation frequency as a function of each gramicidin's concentration. This would be similar to the original studies of Veatch and Stryer (1977), and to Roderick MacKinnon's studies of the voltage-gated K^+ channel (MacKinnon. 1991. *Nature* [*Lond.*] 350:232–235). Unfortunately, the relatively long durations of the hybrid channels makes it difficult to observe enough formation events to unambiguously resolve the dimer versus higher order oligimer question. Given the speculative nature of our assignment of a structure to the hybrid channels, I feel it is safest to begin by considering known structures, such as the intertwined-helical dimer.

RONALD KABACK: Molecular biologists often assume that if function is preserved by a mutation, then the mutation did not grossly perturb the protein molecule's structure. In your paper, however, you describe mutations that radically alter structure but leave function intact. Do you think that this result is relevant to molecules that are larger than gramicidin and, thus, have many more "structure preserving" intramolecular interactions?

DURKIN: That is a very good point. It is clear that the gramicidins exhibit a large degree of structural polymorphism because they are relatively small molecules and, therefore, many conformations contain enough intramolecular interactions to be stable. Even so, a large number of sequence substitutions do not significantly alter gramicidin's membrane structure. On the other hand, I do not know where to draw the line between "small" and "large" molecules. Many proteins fold in apparently independent structural domains that are relatively small, for example, the inactivation peptide of the *Shaker* K^+ channel. It is possible that among a group of mutant proteins a particular domain will exhibit structural polymorphism.

SONIA HOCHERMAN: I have two questions. First, is the intertwined-double helix more stable than the $\beta^{6.3}$-helical dimer? Second, do the two structures have different luminal diameters?

DURKIN: The difference in stability between intertwined- and $\beta^{6.3}$-helices depends on the gramicidin's amino acid sequence and on the environment in which the gramicidins are examined. For example, the most stable conformation of the "parent" gramicidin, [Val^1]gramicidin A, changes from intertwined helices in organic solvents to $\beta^{6.3}$-helices in the membrane. With respect to your second question, the intertwined- and $\beta^{6.3}$-helical dimers have very similar overall lengths and pore diameters. In fact, because of these similarities, a great deal of effort was directed at distinguishing between these models.

HOCHERMAN: Does the difference in the proposed luminal diameters of the intertwined- and $\beta^{6.3}$-helical dimers provide an explanation for their different conductances?

DURKIN: It is difficult to assess the role of the luminal diameter in determining the two channel type's conductances because the channels have different backbone structures.

MURRAY BECKER: Following Ron Kaback's question about structural polymorphism, I would like to address the question of functional polymorphism. You have proposed that the low conductance heterodimers form cation selective, intertwined-helical dimers. However, Bonnie Wallace's CsCl-crystal structure of the intertwined dimer contains anions, quite unexpected for a cation selective channel. Do you believe that the environmental dependence (crystal versus membrane) of channel-ion interactions (cation versus anion) reflects the relatively small size the gramicidin channel, or does it reflect potential problems in using a crystal structure to evaluate the structure-function relation of a membrane protein?

DURKIN: I think that this reiterates a point made many times at this meeting, namely, it may be difficult to determine unambiguously the molecular mechanism responsible for channel function even with hard structural information. With respect to our results, one should remember that the assignment of an intertwined-helical structure to the low conductance heterodimers is speculative. But, if these channels are in fact intertwined-helices, it is not clear whether they need to have a conformation identical to the one found by Bonnie Wallace in gramicidin-CsCl crystals. Other crystal structures of intertwined helices have been reported, and these different crystal structures exhibit a range of channel lengths and helical pitches. Also, as you point out, the crystalline and membrane environments are quite different. All of

these points serve to illustrate potential difficulties in relating a channel's membrane function to its crystal structure.

BECKER: What is the role of the molecule's size in the observed polymorphism? Specifically, in the gramicidin channel the lipid molecules are close to the permeation path. Lipid environmental modulation of, for example, cation versus anion binding may be more important in gramicidin A than in the large protein ion channels.

OLAF ANDERSEN: The gramicidin crystals differ from the more conventional protein crystals by having only $\approx 15\%$ solvent. There is the possibility that the average side chain orientations in the absence of lipid are dominated by packing interactions, which could affect the backbone conformation and, thus (in combination with the altered electrostatics), allow anion binding. That should, however, be a complication that is peculiar to the gramicidin crystals.

DURKIN: We must also remember that the presence of Cl^- in the crystal does not imply that the channels are Cl^- permeable. There is a distinction between Cl^- binding and Cl^- permeation.

ALAN FINKELSTEIN: Do the energy calculations presented in your paper assume that the "wild-type" and "mutant" gramicidins partition equally into the membrane?

DURKIN: If you refer to the channels, $\Delta\Delta G^{\ddagger}$ and $\Delta\Delta G^{\circ}$, are the measure of how well the available peptides "partition" (in a different sense) between the symmetrical channel types and the hybrids. If you refer to the monomers, we do not need to make any assumptions about the monomer concentrations (except that they should vary slowly): as long as both types of monomer are available to make channels, their actual concentrations cancel out. That is what makes this measurement possible, because the monomers are not observable by this method.

ANDERSEN: To see how monomer concentrations drop out, let us consider the various dimers that form when two different gramicidin analogues (A and B) are added to both sides (L = left and R = right) of a planar bilayer. To this end, the channel formation/disappearance reactions are approximated to be a simple monomer $\leftrightarrow$ dimer equilibrium (cf Durkin et al., 1990):

$$A_L + A_R \leftrightarrow AA; \quad K_{AA} = \frac{[A]_L \cdot [A]_R}{[AA]}$$

$$B_L + B_R \leftrightarrow BB; \quad K_{BB} = \frac{[B]_L \cdot [B]_R}{[BB]}$$

$$A_L + B_R \leftrightarrow AB; \quad K_{AB} = \frac{[A]_L \cdot [B]_R}{[AB]}$$

$$B_L + A_R \leftrightarrow BA; \quad K_{BA} = \frac{[B]_L \cdot [A]_R}{[BA]}$$

Now, in the experiments we measure the relative appearance rates, and the relative concentrations, of the two heterodimer types (AB and BA) relative to those of the symmetrical homodimer types (AA and BB):

$$AA + BB \leftrightarrow AB + BA.$$

The equilibrium constant for this reaction is given by:

$$\frac{[AB] \cdot [BA]}{[AA] \cdot [BB]} = \frac{K_{AA} \cdot K_{BB}}{K_{AB} \cdot K_{BA}},$$

which is independent of the monomer concentrations. For further details, see Durkin et al. (1990).

DIANE PAPAZIAN: With a variety of gramicidin peptides, do the structures (or channel types) you see depend on whether you add the peptides to the same or opposite sides of the bilayer?

DURKIN: No, the type of channel that we observe does not depend on which side(s) of the bilayer the peptides are added.

JOSHUA ZIMMERBERG: Are you assuming that the equilibrium between monomers and dimers is the same for both homodimeric channels?

DURKIN: No, our analysis does not assume that the different homodimeric channels have the same free energy for the monomer $\leftrightarrow$ dimer equilibrium. We actually measure the difference between the free energy of heterodimers and the average of the free energies of the two homodimers.

MARCO COLOMBINI: What kind of energy barrier would you expect for an intertwined-helical channel's opening and closing? Can you reconcile it with measured rate constants?

DURKIN: We have not attempted a detailed calculation. Before doing the experiment, I assumed that the dissociation energy for an intertwined-helical dimer would be much higher than for the $\beta^{6.3}$-helical dimer because the former contains many more hydrogen bonds than the latter. However, our experimentally determined activation energies for intertwined- and $\beta^{6.3}$-helical channel dissociation were very similar. In the paper, we reconcile the difference between our intuitive predictions and the experimental results by postulating that the two channel types have different dissociation mechanisms, but that the rate-limiting steps of each of the different mechanisms (coincidentally) have similar activation energies.

H. RICHARD LEUCHTAG: I am intrigued by the topological problem of forming the intertwined-helical dimer. Combining molecular helices sideways requires breaking the backbone in several places and reforming; that seems unlikely. Straightening the monomers out before putting them together also appears unlikely in the absence of a specific enzyme. That leaves putting the helices end to end and screwing them together. But as the screw motion advances, the number of hydrogen bonds increases, making further motion more difficult. Why not settle for a partial overlap of the monomers?

DURKIN: Your presumption is correct, monomer folding is not enzyme catalyzed. A screw mechanism is unlikely because intertwined-helical channels are unable to form when both monomers are only present on opposite sides of the membrane. I believe that the two monomers associate at the bilayer-aqueous phase interface, where they form an intertwined-helical dimer and, after formation, the dimer inserts into the membrane.

BUSATH: Your paper states that tryptophan-bilayer interactions may modulate intertwined- and $\beta^{6.3}$-helical channel formation. Is there any evidence that Trp penetration of the bilayer is unfavorable?

DURKIN: Yes, a gramicidin with a Trp at position 1 (which is near the bilayer's center) forms $\beta^{6.3}$-helical channels, but the partition coefficient is lower than that of the parent channel, gramicidin A.

ANDERSEN: In order to titrate the homodimer channel appearance rates of a gramicidin with a Trp at position 1 and gramicidin A to the same level, you need to add a 300-fold excess of the former gramicidin. Also, compared with gramicidin A and a gramicidin with Phe at position 1, a Trp at position 1 reduces the average channel duration by $\approx$10- and $\approx$5-fold, respectively.

BUSATH: If you replace a tryptophan with a leucine or naphthylalanine, would it form a double helix and, if so, would the substitution's sequence position (i.e., position 9 versus 15) be important?

DURKIN: That experiment has been reported (Koeppe et al., 1991). A peptide was synthesized in which the gramicidin sequence was changed from L-Trp^{9}-D-Leu^{10}-L-Trp^{11}-D-Leu^{12}-L-Trp^{13}-D-Leu^{14}-L-Trp^{15} to L-Leu^{9}-D-Trp^{10}-L-Leu^{11}-D-Trp^{12}-L-Leu^{13}-D-Trp^{14}-L-Leu^{15}. This peptide forms two classes of channels in lipid bilayers: one channel with an average duration of $\approx$100 ms, and another with an average duration near half a minute. Hybrid-channel experiments indicate that the short lived channel is a $\beta^{6.3}$-helical dimer, and there is no energy barrier for heterodimer formation with gramicidin A. The long lived channels do not form heterodimers with gramicidin A and, thus, these channels seem to be intertwined-helical dimers. Therefore, by switching the positions of the Trp and Leu residues, we generate a peptide that can form intertwined- and $\beta^{6.3}$-helical channels with approximately equal ease.

BUSATH: That is remarkable because you have reduced the number of tryptophans by one, but the average sequence position of the Trps along the peptide-chain is unaltered.

DURKIN: That is true, but we must think of the "average" Trp position in a different way. I believe that the important interaction is hydrogen bonding between the nitrogen of the Trp's indole ring and water at the membrane interface. In the $\beta^{6.3}$-helical channel, the position 9 Trp is too far from the interface to form hydrogen bonds with interfacial water. So, we really need to consider the three Trps at sequence positions 11, 13, and 15. The "average" position of these three Trps is different in gramicidin A and in the peptide in which Trp and Leu are interchanged.

ROGER KOEPPE: Further to this question about Trp and Phe, I wish to emphasize that neither gA (with four Trp) nor gM^{-} (with four Phe) can by itself form intertwined-helical homodimeric channels at significant appearance rates. [L-Leu^{9}-D-Trp^{10}-L-Leu^{11}-D-Trp^{12}-L-Leu^{13}-D-Trp^{14}-L-Leu^{15}]gramicidin A is the only known analogue that can do that. The sequence dependence of double-versus single-helical gramicidin channel formation in membranes is not yet understood on theoretical grounds. An explanation of which features in the gramicidin A sequence cause it to form right-handed $\beta^{6.3}$-helical and not left-handed channels is also lacking.

BUSATH: I am concerned about the reversal potential measurements presented in your paper. With a tenfold concentration gradient (or a 7-fold activity gradient), one expects a 52-mV reversal potential, but you report only 42 mV. Do you see the same 10 mV offset with gramicidin A?

DURKIN: In order to resolve the low conducting channels we use the bilayer punch technique, in which a $\approx$25-μm diameter patch of the planar lipid bilayer is isolated with a patch pipette. The unstirred layer and streaming potential effects are more extreme inside a patch pipette. We do see the same offset potential for both the intertwined- and $\beta^{6.3}$-helical channels.

BUSATH: That is small comfort. Couldn't you use an osmotic strength stabilizer to remove the likelihood of water flow across the membrane, or is your offset due to water flowing through the channel?

DURKIN: The offset is due (in part) to water flow through the channel.

ZIMMERBERG: How can you distinguish between the energetics of dimer formation and the energetics of dimer insertion?

DURKIN: Our calculation does not decompose channel appearances into the presumed underlying processes: the dimer's "assembly" and the membrane insertion of the "assembled" dimer. Nevertheless, we unambiguously characterize the overall process, which is the difference in the energetics of homodimer and heterodimer channel formation.

RON KABACK: On that note, I will close this final session and extend our thanks to all those who made presentations and to all those who made this Biophysical Discussion possible.