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On the mechanism of channel-length dependence of gramicidin single-channel conductance

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Single-channel conductance data on four different gramicidin channel lengths demonstrate that conductance magnitude is neither inversely dependent on the square of the channel length nor on the image force arising from differences in the extent of lipid dimpling (Jordan and Vayl (1985) Biochim. Biophys. Acta 818, 416–420). Rather the conductance differences are consistent with the decreased off-rate constant for the singly occupied state as the ionic radius decreases from that of cesium ion to sodium ion coupled with the decreased probability of the doubly occupied channel due to increased ion-ion repulsion as the channel is shortened (Urry et al. (1984) Biochim. Biophys. Acta 774, 115–119).

Previous studies on the effect of changing the channel length of gramicidin A (HCO-Val^1-Gly^2-Ala^3-DLeu^4-Ala^5-DVal^6-Val^7-DVal^8-Trp^9-DLeu^{10}-Trp^{11}-DLeu^{12}-Trp^{13}-DLeu^{14}-Trp^{15}-NHCH$_2$CH$_2$OH) [1] utilized comparison with the shortened channel des-Val^7-DVal^8-gramicidin A (HCO-Val^1-Gly^2-Ala^3-DLeu^4-Ala^5-DVal^6-Trp^9-DLeu^{10}-Trp^{11}-DLeu^{12}-Trp^{13}-DLeu^{14}-Trp^{15}-NHCH$_2$CH$_2$OH) [2]. This shortened analog was chosen as it neither interfered with the sequence of the head to head junction (residues 1 through 5 and the NH of residue 6) nor most importantly with the ion binding site which involves coordination by the carbonyls of residues 9, 11, 13 and 15. On the bases of decreased diffusional length and of the shorter distance over which the potential is applied, an increase in conductance would have been expected if either or both of these effects dominated the ionic mechanism giving rise to conductance. The observation, however, was a sharply reduced conductance from approximately 26 pS to 16 pS for 1 M KCl (0.6 molal activity) at 100 mV, 30 °C and using diphytanoylphosphatidylcholine (DPhPC)/*n*-decane membranes [2]. The result was interpreted in the light of ^{23}Na-NMR ion interaction studies on suspensions of gramicidin A channels in phosphatidylcholine lipid bilayers. These studies demonstrated two ion binding constants: a tight binding constant, approx. 30 M, and a weak binding constant, approx. 1 M^{-1} [3]. Since the channel has two binding sites related by 2-fold symmetry, this meant that the difference between the tight binding constant for single ion occupancy and the weak binding constant for double ion occupancy would be due to ion-ion repulsion on double ion occupancy [3]. The ^{23}Na-NMR studies further demonstrated the singly occupied channel to have a sodium ion off-rate constant of about $3 \cdot 10^5$ s^{-1} which would give rise to an immeasurably small current whereas the sodium ion off-rate constant for the doubly occupied

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channel was $2 \cdot 10^7$ s^{-1} which would give rise to the measured current [4]. Furthermore the NMR-derived binding and rate constants could very satisfactorily be used to calculate the experimental single-channel currents over a significant range of ion activities [5]. The interpretation of the decreased current for 1 M KCl exhibited by des-Val^7-DVal^8-gramicidin A, therefore, was that the measurable current arose dominantly from the doubly occupied channel and that increased repulsion between ions on double occupancy would, for the shorter channel, decrease the probability of the doubly occupied state and hence would decrease the magnitude of the single-channel current [2]. Subsequently an alternative explanation was put forth based on electrostatic modeling of ion pores [6–8] which argued that the shorter channel caused a greater dimpling of the lipid bilayer and that the more imposing lipid surrounding the entrance to the channel would increase 'the electrostatic contribution to the energy barrier near the constriction mouth' and would lead to decreased rate of ion entry into the channel [9].

In order to distinguish between the two proposed mechanisms and to examine the effect of length to a greater extent, cesium ion conductance is considered and additional length analogs (longer channels) have also been examined. These are the elements of this report. The choice of cesium ion is because ^{133}Cs-NMR studies have shown that the singly occupied channel exhibits a very significant off-rate constant greater than $1 \cdot 10^7$ s^{-1} [10] which in contrast to the smaller alkali metal ions would give rise to a very appreciable current. This reduces the argument given for the lower conductance exhibited in the case of potassium ion

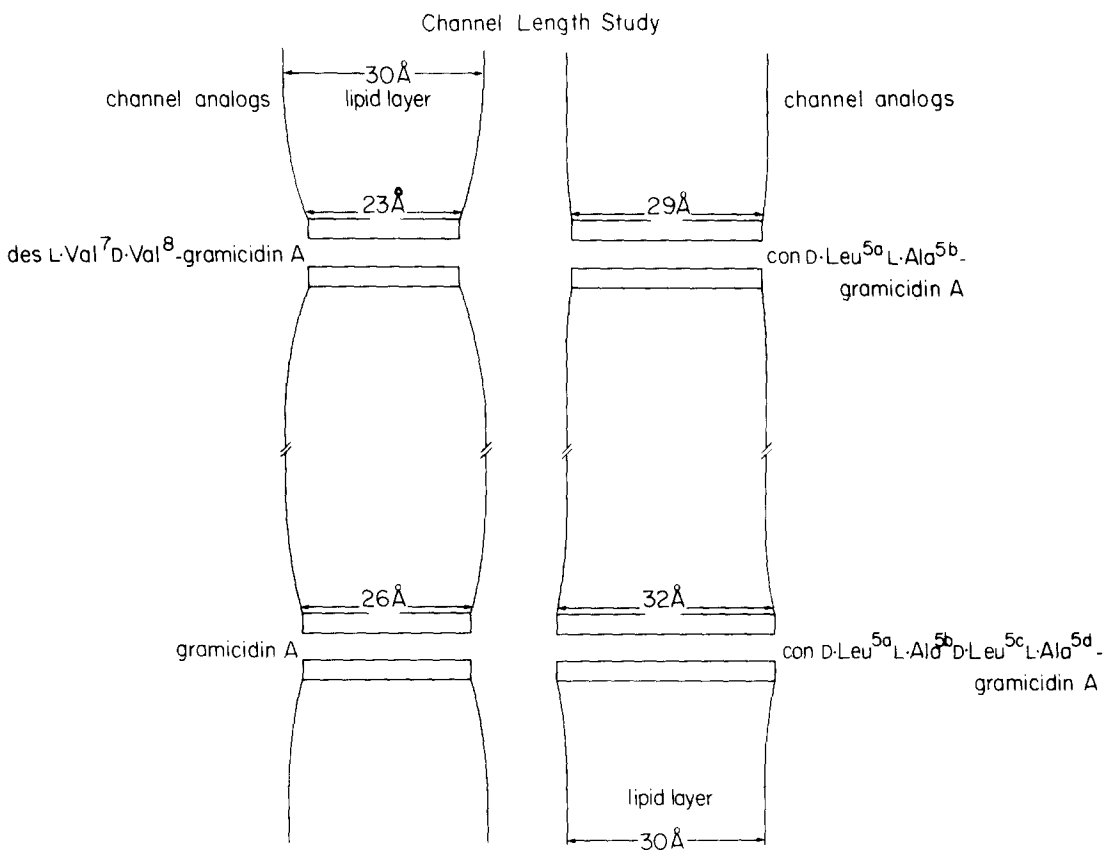

Fig. 1. Schematic representation of gramicidin channel lengths used in this study in relation to a 30Å lipid layer. The approximate mean end to end channel lengths are 23Å for des-Val^7-DVal^8-gramicidin A (analog I), 26Å for gramicidin A, 29Å for con-DLeu^{5a}-Ala^{5b}-gramicidin A (analog II), and 32Å for con-DLeu^{5a}-Ala^{5b}-DLeu^{5c}-Ala^{5d}-gramicidin A (analog III).

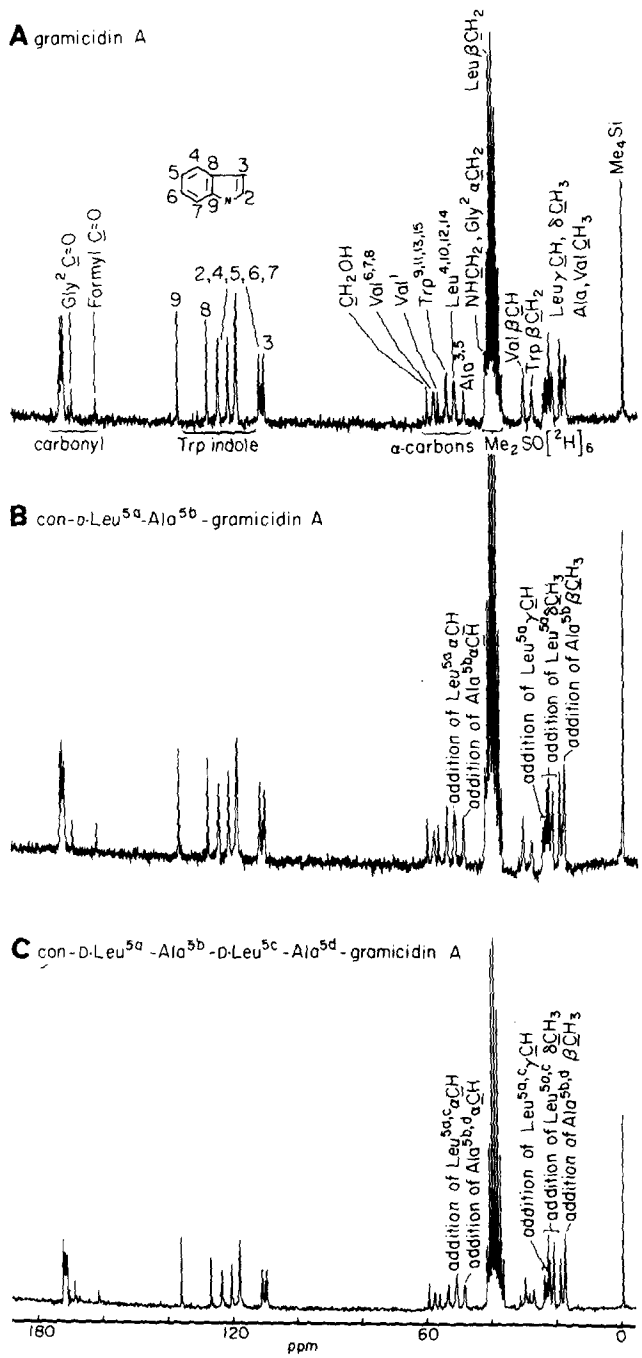

Fig. 2. ^{13}C-NMR spectra: (A) of gramicidin A; (B) of con-DLeu^{5a}-Ala^{5b}-gramicidin A (analog II) and (C) of con-DLeu^{5a}-Ala^{5b}-DLeu^{5c}-Ala^{5d}-gramicidin A (analog III), determined in dimethylsulfoxide-d_6 at 30 °C. The additional resonances due to the added residues are apparent as indicated for the protonated carbons. Expansions of the carbonyl region also demonstrate successful syntheses. At this stage the purity of the sample is greater than 90%; analytical HPLC completes the purification to that required for the lipid bilayer transport studies.

whereas if lipid bilayer dimpling were the basis for the lesser conductance exhibited by potassium ion then this mechanism would continue to be equally operative for the cesium ion. Thus for the cesium ion one expects a more limited conductance decrease on shortening the channel if decreased probability of double ion occupancy were the case for potassium ion [2] whereas essentially the same reduction in conductance is expected if a significant increase in image potential due to lipid dimpling were the explanation of the channel length effect [9].

In this report the study of channel length is also extended to consider longer channels. The length is increased by insertion of one and then two DLeu-Ala dipeptides between residues 5 and 6 in the sequence. These length analogs are designated con-DLeu^{5a}-Ala^{5b}-gramicidin A (II) and con-DLeu^{5a}-Ala^{5b}-DLeu^{5c}-Ala^{5}gramicidin A (III). For these analogs the side chains of the residues in the binding site have the same vicinal, $i \pm 1$ and $i \pm 6$, side chain interactions which would even give rise to the same distribution of side chain rotameric states. The resulting channel lengths are schematically drawn in Fig. 1 in relation to a 30 Å lipid layer thickness. The channels differ in length by 3 Å giving approximate mean lengths of 23 Å, 26 Å, 29 Å and 32 Å. The synthesis of des-Val^{7}-DVal^{8}-gramicidin A (I) has been described and verified previously [2,11] and the syntheses of the longer analogs (II and III) will be given elsewhere (Urry, Trapane and Prasad; Prasad and Urry, in preparation). To verify that the syntheses have indeed been achieved, however, the ^{13}C-NMR spectra of length analogs II and III are presented in Fig. 2 where they are compared to that of gramicidin A. The purity demonstrated in Fig. 2 which is 90% or greater is the starting point for further purification by analytical HPLC. The major peak in the HPLC is clearly the product of interest. A narrow fraction of this peak is collected and again passed through the analytical HPLC. A narrow fraction is again collected and repassed through the HPLC. This procedure is repeated some half dozen times to ensure that the highest level of purity is obtained. The analytical HPLC chromatograms for analogs II and III are given in Fig. 3.

The details of the planar bilayer apparatus, the

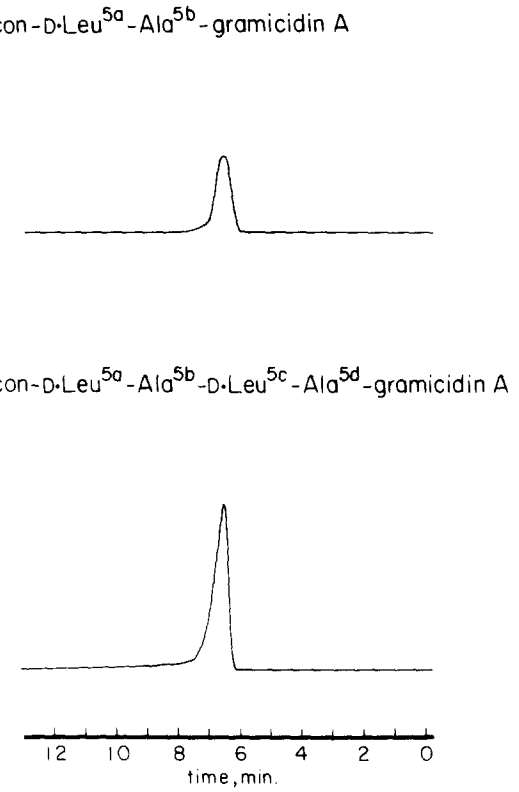

Fig. 3. (A) HPLC of con-DLeu^{5a}-Ala^{5b}-gramicidin A on 'Zorbax' ODS analytical column (4.6 mm×25 cm) using 10% H_2O/CH_3OH solvent system at a flow rate of 1 ml/min. The retention time is 6.5 min. (B) HPLC of con-DLeu^{5a}-Ala^{5b}-Leu^{5c}-Ala^{5d}-gramicidin A on 'Serva' ODS analytical column (4.6 mm×25 cm) using 7.5% H_2O/CH_3OH at a flow rate of 1 ml/min. The retention time is 6.4 min.

data collection and data analyses are as previously presented [2,12] with some minor modifications as recently described elsewhere [13]. The conditions are 0.3 molal activity CsCl, 100 mV, 30 °C and DPhPC/*n*-decane membranes. The histograms of frequency of occurrence vs. single-channel conductance are given in Fig. 4 for the four different channel lengths and the most probable single-channel conductance values are given in Table I. Representative conductance traces are seen in Fig. 5. Apparent in Fig. 5 are the different channel lifetimes which result from the varying mismatch of channel length with membrane lipid layer thickness. This issue, which is one result of the different channel lengths, will be considered elsewhere. What is of particular interest here is

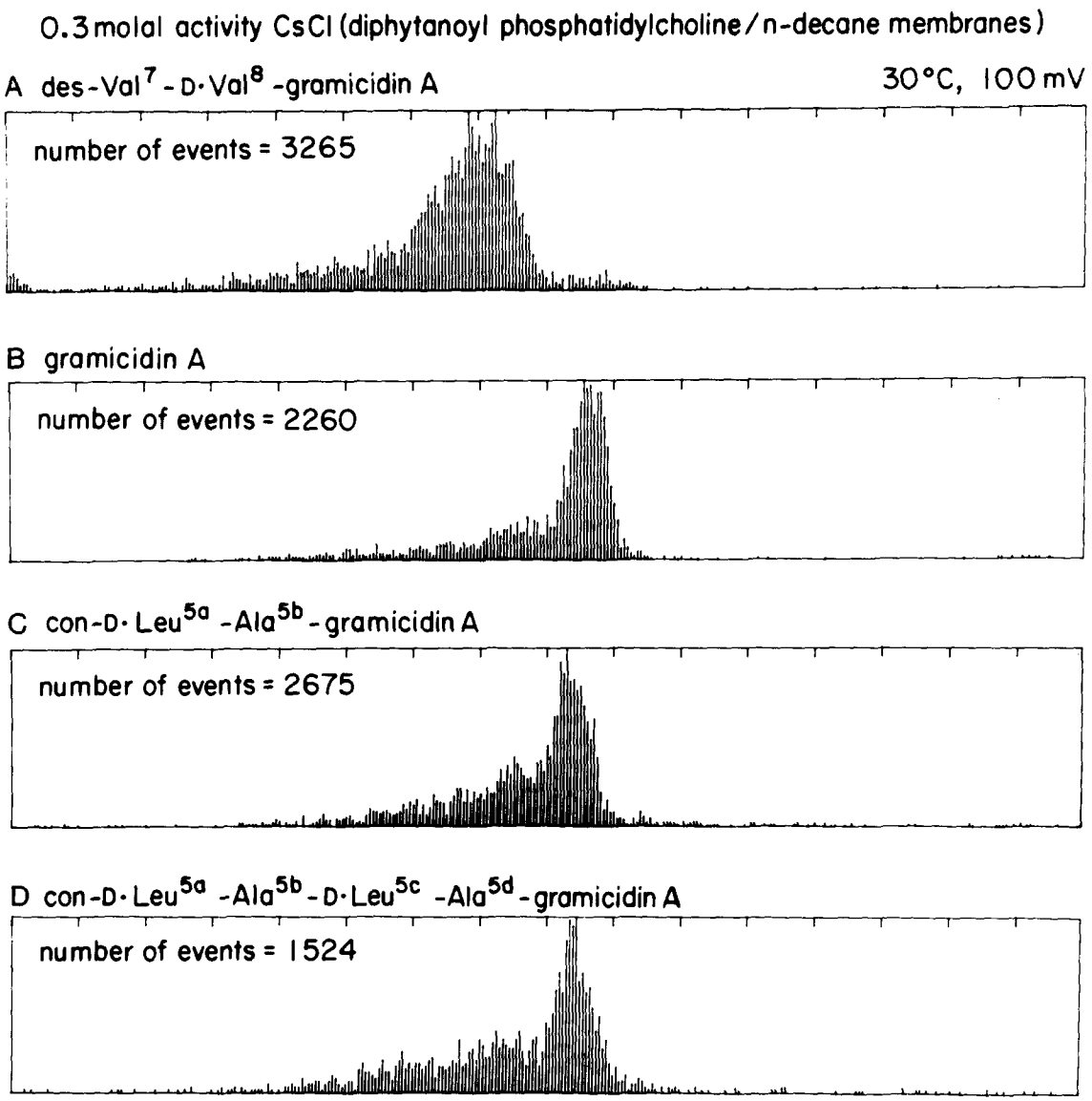

Fig. 4. Histograms of frequency of occurrence vs. conductance magnitude for each of the gramicidin length analogs at 0.3 molal activity CsCl, 30 °C, 100 mV and diphytanoylphosphatidylcholine/*n*-decane membranes. The scaling steps at the top of the histograms are at 5 pS increments. In (A) the mean of the most probable conductance for analog I (des-Val^{7}-DVal^{8}-gramicidin A) is 36 pS; in (B) the mean of the most probable conductance peak for gramicidin A is 43 pS; and in (C) and (D) for analogs II and III, the values are both 42 pS.

that the peaks defining the most probable single-channel conductance values for 0.3 molal activity CsCl are changed very little on changing channel length (see Fig. 4). The shortened channel exhibits a greater dispersity of single-channel conductances; this has been accredited to the greater number of rotameric states available to the side chains of the tryptophan residues in des-Val^{7}-DVal^{8}-gramicidin A [11]. In spite of this the mean of the most probable conductance peak for cesium ion is about 15% lower for the shortened channel than for the other channel lengths whereas for potassium ion the reduction was 38% [2].

The results of Fig. 4 make it quite clear that

TABLE I

MOST PROBABLE SINGLE-CHANNEL CONDUCTANCE FOR DIFFERENT CHANNEL LENGTHS

Analog I, des-Val^{7}-DVal^{8}-gramicidin A; analog II, con-DLeu^{5a}-Ala^{5b}-gramicidin A; analog III, con-DLeu^{5a}-Ala^{5b}-DLeu^{5c}-Ala^{5d}-gramicidin A.

	Molal act.	Analog I (~ 23 Å)	Gramicidin A (~ 26 Å)	Analog II (~ 29 Å)	Analog III (~ 32 Å)
Cs^+	0.3	36	43	42	42
K^+	0.6	16	26		
Na^+	0.6	7	12		

mismatch of channel length and lipid-layer thickness is not a significant factor in altering the magnitude of single-channel conductance and therefore is not responsible for the decreased conductance exhibited by analog I when the permeant ion is potassium. In the previous argument pointing to the decreased probability of double ion occupancy as being responsible for lower conductance exhibited by analog I with potassium ion [2], NMR data on sodium ion interactions was utilized [3–5]. To make the argument more rigorous one should have either the NMR data at low potassium ion concentration or the sodium ion conduc-

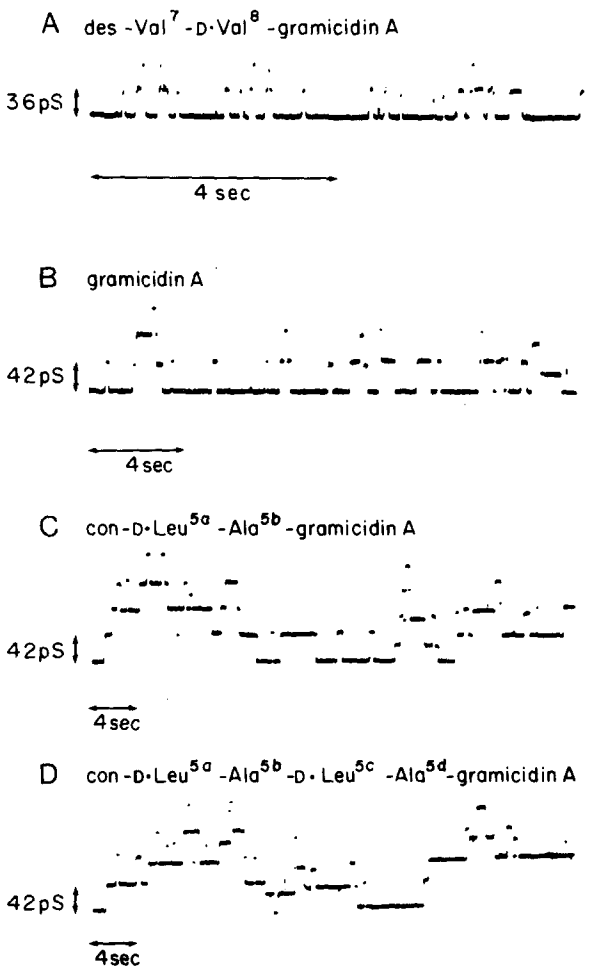

Fig. 5. Conductance traces for the different channel lengths. In (A) for the shortened analog the time for the total trace is 8 s and channel lifetimes are very short; in (B) for gramicidin A the time for the trace is 20 s and the channel lifetimes are longer; and in (C) and (D), the time is 40 s and the channel lifetimes are longer yet. The progressively increasing channel lifetime as the channel length is increased is the result of the different channel lengths. As the channel length approaches the lipid layer thickness, the channel lifetime increases; this demonstrates that different channel lengths are indeed being examined. In this rather course display the conductance steps are all approx. 40 pS.

tance data. Since the former has the greater technical difficulties, the sodium ion conductance data is reported here for analog I. The most probable conductance at 0.6 molal activity, 30 °C, 100 mV, DPhPC/*n*-decane is approx. 7 pS for the shortened analog and 12 pS for gramicidin A. The reduction in sodium ion conductance on shortening the channel is 42%. The sodium ion conductance reported here for gramicidin A is similar to that obtained by the Bamberg and Läuger group for other phosphatidylcholine membranes [14,15]. The decrease in conductance due to shortening the channel is observed for sodium ion to be even greater than for potassium ion (see Table I). The arguments developed from the ^{23}Na-NMR data therefore are directly applicable and directly contrary to the viewpoint that 'not more than one sodium ion occupies a gramicidin A channel at any instant' [16]. In order to rationalize the decreased conductance of the shorter analog with the viewpoint that no more than one sodium ion occupies the channel, it might be argued that the central barrier is increased in the shorter analog. By design, however, the shorter analog provides the interesting case where the first six residues counting from the amino end are identical to those of gramicidin A (interestingly so are the first seven residues counting from the carboxyl end with both counts ending in the common D-valine residue). This means that the two central turns of helix of the channel (the central 10 Å) are identical. Thus it is difficult to imagine that the central barrier is greater for the shorter analog. Furthermore since the difference in conductance on changing channel length is markedly ion specific, the image potential argument, which as presented would have no ion specificity [6–9], is not operative. These results in which the channel length is changed and the membrane is held constant are consistent with those of Hladky and Haydon [17] where the channel was kept constant and the membrane lipid layer thickness was varied. They found the single-channel conductance to be essentially independent of lipid layer thickness.

That the longer analogs exhibit essentially the same cesium ion conductance at 0.3 molal activity is a further demonstration that channel length is not a significant factor at this ion activity. If the dominant factor were diffusional length, then the relative conductances of analogs I and III would compare as $(23)^{-2}$ compares to $(32)^{-2}$, that is, analog I would be expected to exhibit almost twice the conductance of analog III. This clearly discounts diffusional length as a discriminating factor at 0.3 molal ion activity. This is not to imply that the effect of length would not be relevant under other conditions. As the ion activity is increased, saturation of single-channel conductance can occur. In the two site three barrier model, initially and extensively considered by Hladky, Haydon and Urban [18–22] for the gramicidin A channel and also considered along with more complex single filing models by Eisenman, Sandblom and co-workers [23–25], double ion occupancy can become limited at high ion activities by the rate at which the intrachannel ion translocation occurs, that is, by the rate at which the cation in the binding site on the positive side of the membrane jumps to the second ion binding site on the negative side of the membrane. While there are complications at very low ion activity [13], NMR studies have demonstrated two localized binding sites and have directly determined four of the five rate constants required to describe two site three barrier single file channel transport and dielectric relaxation studies have been utilized to obtain information on the fifth rate constant, that is, on the rate over the central barrier [5,13]. Preliminary studies, as yet fragmentary, suggest that for a given ion the four NMR-derived rate constants are not greatly different for the length analogs. This allows that differences in the rate over the central barrier, resulting from the different distances between the two binding sites, may become apparent as shifts in the ion activity at which maximal conductance is achieved. Studies to detail these effects are underway.

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