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SHORTENED ANALOG OF THE GRAMICIDIN A CHANNEL ARGUES FOR THE DOUBLY OCCUPIED CHANNEL AS THE DOMINANT CONDUCTING STATE

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A shortened analog of the gramicidin A transmembrane channel has been synthesized and its transport characterized in planar lipid bilayer membranes. General considerations of a shorter diffusional length and a shorter distance over which the voltage drop occurs (i.e., an increased electric field) would contribute to an increase in single-channel conductance. The finding of a decreased single-channel conductance supports the perspective that the dominant conducting state is the doubly occupied channel wherein distance-dependent repulsion due to the first ion in the channel impedes entry of the second ion in the shorter channel.

Gramicidin A has the primary structure (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}-$NHCH_2CH_2OH$) [1]. The channel structure, that of two left-handed, single-stranded $\beta^{6.3}$-helices hydrogen-bonded amino end to amino end [2–7], is shown in wire model in Fig. 1A along with a plot of the ion-induced carbonyl carbon chemical shifts [7–9]. In fig. 1B is the reported Gibbs free energy profile for sodium ion passage through the channel [10]. Figure 1 contains the essential information for a choice of a shortened analog and for a resulting discussion of ionic mechanism. The binding sites utilize residues 9 through 15 of the sequence; the head to head junction for dimer formation utilizes residues 1 through 5 and the NH of residue 6. This leaves the definition of residues 6, 7 and 8 as the midsection. The gramicidin A channel can be shortened, keeping both the ion binding sites and the head to head junction intact, by synthesizing the des-Val^7-DVal^8-gramicidin A analog (equivalently des-DVal^6-Val^7-gramicidin A). In such an analog, the binding constant and the off rate constant for single-ion occupancy should be essentially unchanged.

The des-Val^7-DVal^8-gramicidin A analog was synthesized by the solid-phase method of peptide synthesis [11]. Since the repeated acid deblocking step involved in the synthesis was known to lead to the destruction of tryptophan residues, a formyl group for the protection of the β-indole ring [12,13] was used in the current synthesis. The synthesis was carried out on a Vega 250C peptide synthesizer (Vega Biochemicals, Tucson, AZ) with a program as outlined previously from this laboratory [14,15]. The N-terminal protected peptide was removed from the resin by ethanolamine treatment which simultaneously deblocked the formyl protection of the tryptophan residues. After deblocking the N-terminal protecting group, the desformyl-des-Val^7-DVal^8-gramicidin A analog was formylated and further purified by high-performance low-pressure liquid chromatography (HPLPLC) [16] on a preparative scale. After checking on an analytical high-performance liquid chromatography (HPLC) column, the fractions were combined

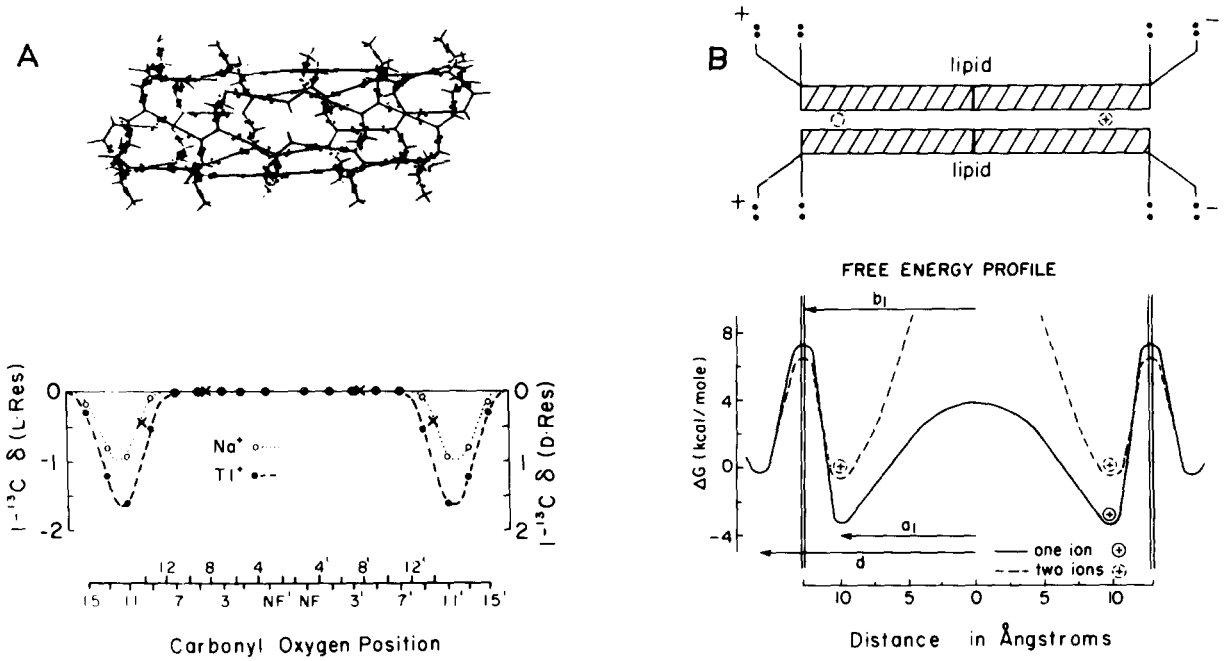

Fig. 1. (A) Above: Wire model of the gramicidin A transmembrane channel which is comprised of two left-handed, single-stranded β-[6,3] helices hydrogen-bonded head to head (amino end to amino end). Below: Aligned with the wire model is a plot of the ion induced carbonyl carbon chemical shifts showing the location of the two ion binding sites. With a translation along the helix axis of 1.53 Å per LD-dipeptide and with the head to head junction the distance between binding sites is approximated as 23 Å. Reproduced from Ref. 8 with permission. (B) Above: Schematic representation of channel spanning the lipid layer of a membrane across which is applied a voltage. Below: Gibbs free energy profile for passage of sodium ions through the gramicidin A transmembrane channel. Using the information in part A the distance a_1 from center to binding site would be 11.5 Å for gramicidin A and 10.0 Å for the shortened analog. The distance b_1 is taken as 2.5 Å longer than a_1 in both cases. Reproduced from Ref. 10 with permission.

and the des-Val[7]-DVal[8]-gramicidin A analog was lyophilyzed from a methanol/water mixture.

Verification of the synthesis and purity of the des-Val[7]-D-Val[8]-gramicidin A analog was checked by ^{13}C-NMR and ^{1}H-NMR. A ^{13}C-NMR spectrum of the synthetic product in dimethylsulphoxide-d_6 is given in Fig. 2 along with a comparable spectrum for the commercially available natural gramicidin A mixture. The spectra were collected at 30 °C on a JEOL PFT-100 spectrometer operating at 25 MHz. A comparison of the assignments for the natural material [14,17] with the synthetic analog shows that there is the expected loss of intensity in the valine α- and β-carbon resonances. Note the absence of any extraneous signals in Fig. 2B which indicates the purity of the synthetic analog to the level of NMR detection (> 95%). ^{1}H-NMR spectra (not shown) similarly verify the synthesis and purity.

Having established purity to greater than 95%, the sample is passed through an analytical HPLC column. The major peak, which is unmistakably the desired sample, is collected, passed through the analytical HPLC column several times; and in a final pass a fraction of the major peak is collected for the planar bilayer studies. This assures the highest level of purity and is the procedure used both for synthetic [1-^{13}C]Val[1]-gramicidin A and for des-Val[7]-DVal[8]-gramicidin A which are compared in this report. Planar black lipid membranes were formed using the methods previously described [18,19]. The lipid used to form the membrane was diphytanoylphosphatidylcholine 2% (w/v) in *n*-decane and the bathing solution was 1 M KCl. The experimental set up consisted of a Teflon cell (aperture 0.6 mM), which is contained in a metal box for better shielding, with a Keithley 427 current amplifier mounted on a Micro-*g* vibration free table. The signal coming from the cell was amplified and passed through a Krohn-

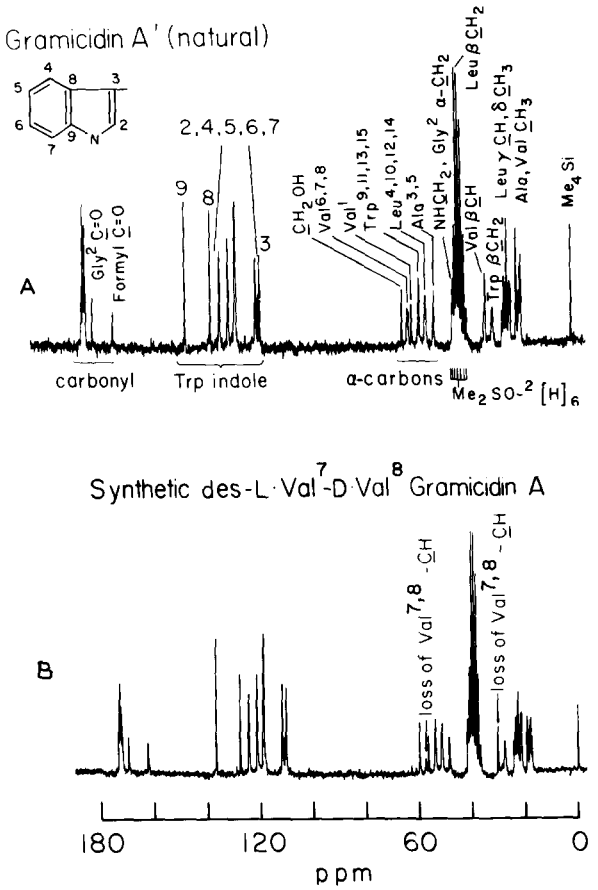

Fig. 2. ^{13}C-MNR spectra at 25 MHz in dimethylsulphoxide-d_6 at 30 °C for (A) natural gramicidin A and (B) synthetic des-Val^7-DVal^8-gramicidin A.

Hite filter, model 3342, set in the Low Pass RC mode. The current amplifier rise time was set to the fastest position (0.01 ms) so the system response to the incoming signal was determined by the Krohn-Hite filter setting (200 Hz, sampling interval = $1/(2 \times \text{freq.})$) within the limits of the current amplifier band-pass. The slew rate of the current amplifier was measured to be about 10 V/0.5 ms in the 10^{11} V/A gain position. The rise time of characteristic channels varied in the range 8–15 ms at 31 °C and 200 Hz. The temperature of 31 °C was chosen as a reasonable balance between short channel lifetime and magnitude of single-channel conductance.

The histogram for the frequency of occurrence of single-channel conductances for des-Val^7-DVal^8-gramicidin A is given in Fig. 3A where it is

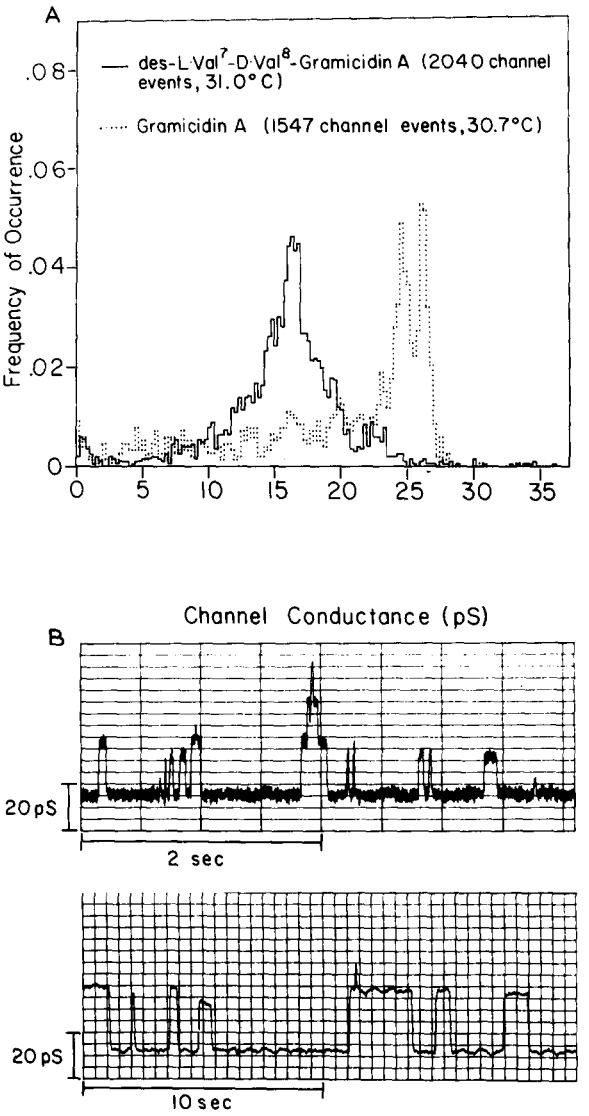

Fig. 3. (A) Single-channel conductance histograms for des-Val^7-DVal^8-gramicidin A (solid line) and for synthetic [1-^{13}C]Val^1-gramicidin A (broken line). The histograms are automatically constructed by computer using an algorithm that optimally matches the downward and upward transitions to capture single-channel events, and only those transitions that can be satisfactorily matched are included in the histograms. The conductance band width is 0.25 pS. (B) Typical conductance trace obtained for des-Val^7-DVal^8-gramicidin A. With a sampling time of 1 ms per data point, the sweep consisting of 4096 digitized data points spans a time window of 4.095 s. (C) A conductance trace for [1-^{13}C]Val^1-gramicidin A. Here the sampling time is 5 ms per point covering a window of 20.475 s.

compared to that of gramicidin A. Also seen in Figs. 3B and C are the conductance traces where it is apparent that the channel lifetimes for des-Val^7-DVal^8-gramicidin A are much shorter. This interesting aspect of decreased lifetime will be considered elsewhere along with longer analogs of the gramicidin A channel. In this effort, our concern is with the magnitude of the single-channel conductance which makes it necessary to establish that the lesser conductances of the shorter channel do not arise from limitations in instrument response time. That the instrument rise times are sufficiently fast is considered above. Here the same is demonstrated by noting that the most probable states of the histograms are identical when comparing those derived from channel events with lifetimes of less than 150 ms with those of greater than 150 ms. Thus the comparison of histograms is not affected by the differences in mean channel lifetimes. This brings the discussion to the issue of why the conductance magnitude is so much less for the shortened channel.

With a shortened channel, the effective electric field is increased for the same 100 mV applied potential and the diffusional length is decreased. These changes are of a nature that would lead to increases in the magnitude of the single-channel conductances. Yet what is actually observed for des-Val^7-DVal^8-gramicidin A is a decrease in the mean conductance and in the conductance of the most probable conducting state (see Fig. 3) from about 26 pS for gramicidin A to about 15 pS for the shorter channel. In the treatment according to Urban, Hladky and Haydon of transport through the gramicidin A channel based on a phenomenological fitting of the single-channel conductance data using a two-site model [20,21] and in our calculation of single-channel conductances using nuclear magnetic resonance (NMR) derived rate constants for sodium ion [10], the off-rate constant for the singly occupied channel is two orders of magnitude slower than that for the doubly occupied channel. This means that the doubly occupied channel would be overwhelmingly the dominant conducting state. In recent NMR characterization of sodium ion binding to the des-Val^7-DVal^8-gramicidin A channel, the binding constant for single occupancy is essentially the same as for gramicidin A which means that the binding site is essentially unchanged and knowing the structure and binding site information that the off-rate constant for single occupancy would be essentially unchanged (unpublished results). On the other hand, the binding constant for going to double occupancy is reduced substantially.

For the two-site channel in which the dominant term i'_{xx}, in the current equation is due to the doubly occupied state one can write [10],

$$i'_{xx} \equiv \chi_{xx} k^{w}_{off} e^{lzFE/2dRT} \quad (1)$$

It is appreciated that there is the reverse flow in which state *xo* goes to *xx* but for net flow to the right *xx* going to *xo* is the dominant process and as noted above, this is very much larger than *ox* going to *oo*. To avoid any confusion, Eqn. 1 is given as a definition. Following Eisenman and colleagues [22–24], χ_{xx} is the probability of the doubly occupied channel and, following our previous nomenclature [10,25], k^{w}_{off} is the rate constant for an ion leaving the weak site, i.e., leaving the doubly occupied channel. The Eyring factor, $\exp(lzFE/2dRT)$, is the effect of the applied field over the distance, $l = b_1 - a_1$, from the binding site to the exit barrier for a channel of total length $2d$ (see Fig. 1). F is the Faraday, E the applied potential and R the gas constant. Clearly from this factor, decreasing $2d$ but leaving l the same length, as occurs in the shortened analog, would lead by the Eyring factor to an increase in conductance to a value greater than 26 pS. If a diffusional process were dominant, i.e., if ion movement through the channel were considered to be a one-dimensional random walk process, then passage through the channel would have an approximate inverse dependence on the square of the distance. Again conductance of the shorter channel would become substantially greater than 26 pS. As the experimental result is instead a decrease by about 50%, the explanation must arise from an aspect of the ionic mechanism that is not considered in the above general arguments.

It is the general view that the current passing through the gramicidin A channel is limited by the rate of ion entry into the channel [10,20,21,26]. Ion entry into an empty channel would be subject to the above arguments with no obvious means of resolving the indicated paradox. Entry of a second

ion into a singly occupied channel, however, as has been suggested to occur [10,20,21–24], would depend on the repulsion between ions. Given the binding sites as defined in Fig. 1, the difference in repulsion energy, δE, due to the decreased distance between sites occupied by monovalent cations can be expressed as

$$\delta E = \frac{e^2}{r_2 \epsilon_2} - \frac{e^2}{r_1 \epsilon_1} \tag{2}$$

where $e^2 = 627\, a_0$ kcal/mol with $a_0 = 0.529$ Å; r_1 and r_2 are the distance between binding sites of 20 Å and 23 Å for the shortened analog and for gramicidin A [8,9], respectively; and ϵ_1 and ϵ_2 are the respective interceding dielectric constants. Taking an average value of ϵ to be 5, as can be estimated from the difference in binding constants for the first and second ion in the channel [10,27], this gives an increase in repulsion energy of about 0.43 kcal/mol, i.e., about 0.73 RT. This quantity is sufficient to reduce the current by the amount observed, i.e., by a factor of 1/2. The result suggests that the conducting state is the doubly occupied channel. Equivalently the result argues that diffusional length between sites is not the dominant factor to consider in gramicidin A channel transport but rather it is the barrier heights for going from one site to another that controls the rate of ion transport through the channel, that is, the process is well-described at this level by Eyring rate theory.

The spread of the conductance for the shortened analog, apparent on comparison with gramicidin A in Fig. 3, is considered elsewhere [28].

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