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Induction of Conductance Heterogeneity in Gramicidin Channels†

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ABSTRACT: In previous work from our laboratory, 5–10% of the channels formed by [Val^1]gramicidin A have conductances that fall outside the narrow range that conventionally has defined the standard gramicidin channel [e.g., see Russell et al. (1986) *Biophys. J. 49*, 673]. Reports from other laboratories, however, show that up to *50%* of [Val^1]gramicidin channels have conductances that fall outside the range for standard channels [e.g., see Prasad et al. (1986) *Biochemistry 25*, 456]. This laboratory-to-laboratory variation in the distribution of gramicidin single-channel conductances suggests that the conductance variants are induced by some environmental factor(s) [Busath et al. (1987) *Biophys. J. 51*, 79]. In order to test whether extrinsic agents can induce such conductance heterogeneity, we examined the effects of nonionic or zwitterionic detergents upon gramicidin channel behavior. In phospholipid bilayers, detergent addition induces many changes in gramicidin channel behavior: all detergents tested increase the channel appearance rate and average duration; most detergents decrease the conductance of the standard channel; and all but one of the detergents increase the conductance heterogeneity. These results show that the conductance heterogeneity can result from environmental perturbations, thus providing a possible explanation for the laboratory-to-laboratory variation in the heterogeneity of gramicidin channels. In addition, the differential detergent effects suggest possible mechanisms by which detergents can induce the conformational perturbations that result in gramicidin single-channel conductance variations.

An important problem pertaining to understanding membrane protein function is how function depends on the membrane environment in which a protein resides. This is a difficult problem to address in studies on populations of membrane proteins that reside within a sometimes rather poorly controlled native lipid environment. Single-channel studies on membrane-spanning channels in planar lipid bilayers, however, allow the functional consequences of lipid environment modulations to be studied in detail. Single-channel experiments are particularly useful to examine questions pertaining to the possible heterogeneity among membrane proteins, because one by definition studies single molecular assemblies (cf. Figure 1). The cation-selective channels formed by the linear gramicidins are in fact exquisitely sensitive to subtle perturbations of the host bilayer.

The linear gramicidins are pentadecapeptides that have the sequences (Gross & Witkop, 1965) HCO-L-Xxx---Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val-D-Val-L-Trp-D-Leu-L-Yyy-D-Leu-L-Trp-D-Leu-L-Trp-$NHCH_2CH_2OH$, where Xxx can be Val or Ile and Yyy can be Trp (in gramicidin A), Phe (in gramicidin B), and Tyr (in gramicidin C). The predominant form is [Val^1]gramicidin A. The sequences are extremely hydrophobic (Segrest & Feldman, 1974). In lipid bilayers, they form $\beta^{6.3}$-helical dimers that act as cation-selective ion channels that have measurable conductance and lifetime (Figure 1) [for a recent review, see Andersen et al. (1988)]. Most gramicidin channels have conductances that fall within a narrow, approximately Gaussian distribution. Occasionally, a channel has a conductance that is significantly different from that of the standard channels (Busath & Szabo, 1981). Most

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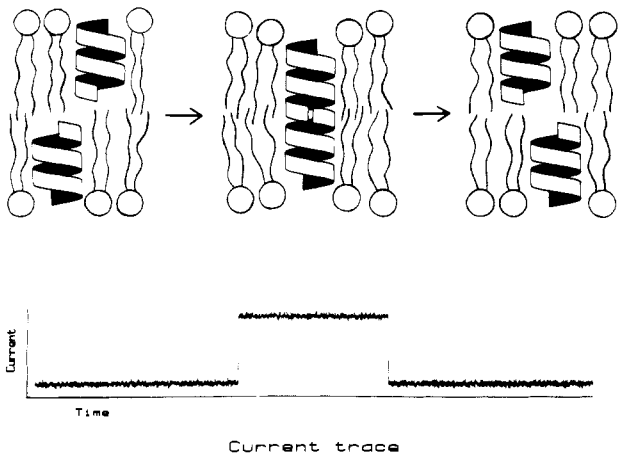

FIGURE 1: Schematic representation of some of the steps in a gramicidin channel appearance/disappearance event. Two gramicidin monomers associate in a bilayer to form an NH_2-terminal-to-NH_2-terminal dimer that is stabilized by six intermolecular hydrogen bonds. The helical dimer forms a channel 2.6 nm long that can span the bilayer. Cation movement through the channel can be recorded as a finite and stable step change in the membrane current that ceases upon dissociation of the dimer. The average duration of the gramicidin dimer is on the order of 1 s.

of these variants have conductances that are less than the standard channel conductance, and they are collectively referred to as "minis". In current transition amplitude histograms, these channels have conductances that are distributed broadly below (and sometimes above) the main peak that corresponds to the standard channels (Busath & Szabo, 1981, 1988a; Busath et al., 1987).

The gramicidin channels have been extensively studied as prototypical membrane-spanning channels. Consequently, much work has been done to elucidate the basis for gramicidin mini channel appearances. In particular, it has been suggested that mini channels may result when one or more tryptophanyls have altered rotameric states, which could alter the librations of neighboring peptide carbonyls and thus the conductance (Urry et al., 1981, 1984c). This was supported by experiments with gramicidin analogues that were modified so as to decrease the number of rotameric states that might be available to the tryptophanyls (Urry et al., 1984c). In other experiments, however, two modifications that should increase the mobility of the tryptophan at position 13 in a similar way have opposite effects on the conductance heterogeneity (Urry et al., 1984b; Prasad et al., 1986). The precise role of carbonyl libration in inducing conductance heterogeneity is thus uncertain.

Results from our and other laboratories show that mini channels cannot be an intrinsic property of the gramicidins. Rather they must result from interaction(s) between the channel-forming peptides and some environmental contaminant(s). Most importantly, the mini frequency for [Val^1]-gramicidin channels varies among laboratories, from ~5% (Mazet et al., 1984; Russell et al., 1986) to about 50% (Urry et al., 1984b; Prasad et al., 1986). This large variation suggests that differences in experimental procedures alter the behavior of gramicidin channels. It has, in fact, been shown that the mini frequency depends on the source of lipid, gramicidin, and electrolyte solutions (Busath et al., 1987). This suggests that some, as yet unidentified, contaminant(s) in one or more of the solutions is (are) the mini-inducing culprit, i.e., that mini channels appear as the result of environmental perturbation(s) of the channel structure.

Here we show that detergents, when added in low concentration to the aqueous phase, can cause a significant increase in mini frequency. Detergents have other effects on single-channel parameters, which we relate to their properties as surface-active agents. The ability of detergents to induce mini channels may account for the large interlaboratory variation of mini channel frequency. These results also suggest a possible mechanism for mini channel formation. Some of this material has appeared in a preliminary form (Sawyer et al., 1988).

Materials and Methods

Diphytanoylphosphatidylcholine (DPhPC)[1] and dioleoylphosphatidylcholine (DOPC) and -phosphatidylserine (DOPS) were from Avanti Polar Lipids (Birmingham, AL), and *n*-decane was from Wiley Organics (Columbus, OH). Glyceromonoolein (GMO) was from Sigma (St. Louis, MO). Gramicidin D, a mixture of gramicidins A, B, and C, was also from Sigma Chemical Co. Ethanol was from U.S. Industrial Chemicals (Tuscola, IL). NaCl was Suprapur grade from E. Merck (through MCB, Cincinnati, OH), HCl was 1.0 N from J. T. Baker Chemical Co. (Phillipsburg, NJ), and water was deionized Millipore Corp. Milli-Q water (Bedford, MA). The DPhPC was further purified by ion-exchange chromatography (Andersen, 1983), and the NaCl was roasted at 500 °C for 24 h and stored over NaOH in evacuated desiccators. [Val^1]Gramicidins A, B, and C were purified from the commercial mixture as described previously (Koeppe & Weiss, 1981).

Detergents from several sources were used. Triton X-100, purity >98%, was from Sigma; protein-grade material was from Calbiochem (La Jolla, CA) or from Pierce Chemical Co. (Rockford, IL). As monitored by fluorescence spectroscopy (excitation at 280 nm), the two protein-grade materials each contained a different contaminant. Both contaminants seemed to be present in the 98% pure material. (We thank Dr. Suzanne Scarlata for doing these measurements.) Reduced Triton X-100, Genapol X-100, and Zwittergent 3-12 (all protein grade) were from Calbiochem. Octyl β-glucoside was from Sigma (through S. Scarlata). The detergents were dissolved in water to make suitable stock solutions. Lysolecithin was monopalmitoylphosphatidylcholine from Avanti and was dissolved in ethanol.

Except where noted, all glassware was washed with dichromate–sulfuric acid and rinsed with Millipore water. "Detergent-washed glassware" was borrowed from a laboratory that uses standard glassware cleaning procedures.

Planar lipid bilayers were formed by the pipet technique of Szabo et al. (1969) across a hole (diameter ~1.6 mm) in a Teflon partition separating two identical Teflon chambers. The general experimental procedures were as described in Andersen and Muller (1982) and Andersen (1983). The membrane-forming solution was, unless otherwise specified, diphytanoylphosphatidylcholine (DPhPC) in *n*-decane (2.5% w/v). The experiments were done with symmetrical unbuffered electrolyte solutions (unless otherwise specified, the electrolyte was 1.0 M NaCl). The temperature was maintained at 25 ± 1 °C by water circulating in an external jacket.

Aliquots of doubly HPLC purified gramicidins (A, B, or C), dissolved in ethanol (concentration ~10 nM), were added in equal amounts to both aqueous phases during vigorous stirring. The stirring was continued for more than 1 min before the measurements began. The nominal concentrations in the

[1] Abbreviations: DOPC, dioleoylphosphatidylcholine; DOPS, dioleoylphosphatidylserine; DPhPC, diphytanoylphosphatidylcholine; GMO, glyceromonoolein; HPLC, high-pressure liquid chromatography; CMC, critical micellar concentration(s); CPK, Corey–Pauling–Koltun.

electrolyte solutions were ~10 pM. (In a few experiments, [Val^1]gramicidin A was included in the membrane-forming solutions, at a peptide/lipid molar ratio of $1/10^7$.)

In this study, all single-channel measurements were done at 100-mV applied potential (200 mV in experiments with gramicidin B). By use of the bilayer punch technique, a "small" membrane was isolated with a glass pipet (diameter ~30 μm) from the "large" membrane that was formed across the hole in the Teflon partition (Andersen, 1983). Single-channel current transitions were detected, and their amplitude was determined as described in the same article. Briefly, the magnitude of single-channel current transitions caused by gramicidin association or dissociation was determined as the difference between the average current level in 40 ms long records collected just before and just after each current transition. In transition histograms, no attempt was made to match channel appearance events with channel disappearance events. To ensure that low-amplitude current transitions would be incorporated into the sample, the discrimination level that triggers "channel" detection was set sufficiently low that base-line current noise would trigger a significant number of "transitions". This procedure also allowed us to obtain an estimate of the instrumental noise. Each experiment consisted of 5–10 consecutive measurements based on 500 current transitions, where 200–400 typically were resolved and displayed as current transition histograms (e.g., Figure 4). Records were eliminated from the final data set if there was poor small membrane stability, if the channel activity was too high, or if there was evidence for increased series resistance originating in the pipet.

Determinations of current transition amplitudes and mini channel frequencies were based on current transition histograms. However, the experiments were also analyzed by using current amplitude histograms (displayed in Figure 5) that were constructed by sampling the membrane current every 2 ms and incrementing the bin corresponding to the current reading. These histograms provide information about the amount of time that is spent at diffeent current levels, and thus about the channel current amplitudes, but they do not provide information about how many channel appearances underlie any given peak.

After collection of results to estimate the single-channel parameters in the absence of detergent, small aliquots of the desired detergent were added in equal amounts to both electrolyte solutions during vigorous stirring that was continued for more than 2 min to allow the detergent to equilibrate with the membrane before the measurements were resumed. The detergent addition led to a large increase in channel activity (see Results), often to a level that was too high to obtain reliable estimates of the single-channel parameters. When this occurred, channel activity was reduced by repeatedly reforming the large membranes until the activity became appropriate.

The magnitude of the detergent-induced increase in channel activity was measured by recording the conductance of the large membrane from which the small membranes were "punched" out for the single-channel measurements. This was done by recording the membrane current response to small (≤20 mV) applied potential differences.

The distinction between normal and mini current transitions was based upon the criteria outlined in Busath et al. (1987), and the mini frequency was calculated as the number of events more than 3 standard deviations from the average current value for the main peak divided by the total number of events in the current transition histogram, multiplied by 100. To facilitate

FIGURE 2: Single-channel activity induced by gramicidin A in DPhPC/decane membranes before and after addition of 8 μM Triton X-100 (Calbiochem). (Top) Control, before Triton addition. (Bottom) Results obtained after Triton addition (this trace was recorded ~3 min after the top trace). Channel currents are upward. In both traces, note the uniform amplitude of most channels. Some mini channels are denoted by arrows. Addition of Triton X-100 leads to an increased channel appearance rate and channel duration as well as a decreased amplitude of the single-channel current steps and an increased mini channel frequency. Filtered at 100 Hz.

visual inspection, the results are plotted not only as current transition amplitude histograms (that correspond to statistical density functions) but also as accumulation plots: the normalized integrals of the corresponding current transition histograms, i.e., the percentage of events with amplitudes at or below the given current amplitude (these plots correspond to statistical distribution functions). The latter plots are useful to estimate the percentage of events within, as well as outside, the main peak.

Channel durations were determined by matching channel appearances with disappearances and measuring the duration between these. Only events whose amplitude fit within the desired distribution in the amplitude histogram for that experiment were used in the analysis. When channels occurred singly, this was straightforward. When a new channel appeared while one (or more) channels were conducting, channel appearances and disappearances that fell within the limits of the amplitude distribution were matched by using a randomized assignment. This procedure is valid as long as all appearances (and disappearances) of the same amplitude represent the same type of molecular event. Channel durations were plotted as normalized survivor plots [e.g., see Andersen and Muller (1982)]. To estimate the average duration, τ, of the distributions, single-exponential decays, $N(t)/N(0) = \exp(-t/\tau)$, were fitted to the results by using a maximum likelihood estimator (Hall & Sellinger, 1981), where $N(t)$ denotes the number of channels with a duration $\geq t$ and $N(0)$ is the estimated total number of channels in the population. $N(0)$ differs from the number of events actually observed, because the on-line channel detection algorithm will not always define unambiguously events with durations less than 40 ms; such events may thus be excluded in the subsequent analysis.

Results

Addition of the nonionic detergent Triton X-100 to the aqueous phase bathing a gramicidin-doped bilayer induces several changes in channel behavior (Figure 2): the channel activity increases as the result of large increases in the channel appearance rate and stability (the disappearance rate decreases); the single-channel conductance heterogeneity increases (the mini frequency increases); and the amplitude of the most probable current transition decreases.

Quantitatively, the increase in channel activity is the most dramatic effect. Before detergent addition (Figure 2, top), a channel appeared once every few seconds—the average number of open channels was <0.1. After detergent addition (Figure 2, bottom), several channels appeared and disappeared

Table I: Gramicidin A with Triton X-100 from Three Separate Sources

expt	Triton[a]	I (pA) ± SD	N^b	% minis	τ (ms)	N^c
1	–	1.48 ± 0.08	1129	5.2	520	292
	+Calbiochem	1.21 ± 0.05	4469	13.3	3070	255
2	–	1.43 ± 0.06	1053	8.7	450	482
	+Calbiochem	1.24 ± 0.05	2532	51.2	2550	313
3	–	1.40 ± 0.05	1343	5.9	490	778
	+Sigma	1.15 ± 0.04	3182	23.8	3550	600
4	–	1.32 ± 0.03	1133	8.1	530	691
	+Sigma	1.13 ± 0.03	2955	17.4	3010	161
5	–	1.39 ± 0.04	1757	10.9	550	687
	+Pierce	1.22 ± 0.03	2098	11.0	2120	140
6	–	1.38 ± 0.04	2780	9.0	510	1148
	+Pierce	1.22 ± 0.04	2340	12.3	1980	258
7	–	1.45 ± 0.05	1428	4.8	450	347
	+Pierce	1.25 ± 0.04	3467	11.9	3250	727
8	–	1.47 ± 0.05	1536	8.0	700	682
	+Pierce	1.21 ± 0.03	2617	19.2	2640	965
9	–	1.33 ± 0.03	1255	5.2	880	579
	+Pierce	1.17 ± 0.03	2637	9.6	3100	480

[a] 8 μM Triton X-100. [b] Number of events in the aggregate current transition histogram. [c] Number of channels in the aggregate channel duration histogram.

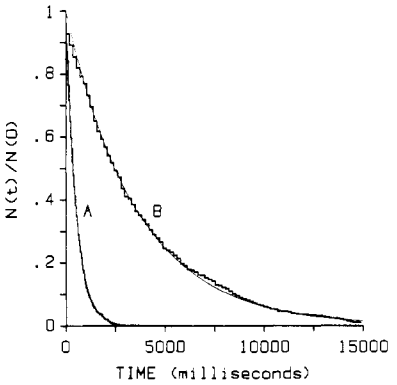

FIGURE 3: Normalized survivor plots of gramicidin A channel durations in DPhPC/decane membranes before and after addition of Triton X-100. (A) Control, before Triton X-100 addition; 778 channels from the main peak of amplitude histograms (see Figure 4) of 6 consecutive measurements on a single day. (B) Results after addition of 8 μM Triton (Sigma); 600 channels from the main peak of amplitude histograms of 4 consecutive measurements taken on the same day as (A). The curves denote a fit of a single-exponential decay to the results: control τ = 490 ms [$N(0)$ = 885] with 8 μM Triton, τ = 3550 ms [$N(0)$ = 640]. Experiment 3 of Table I.

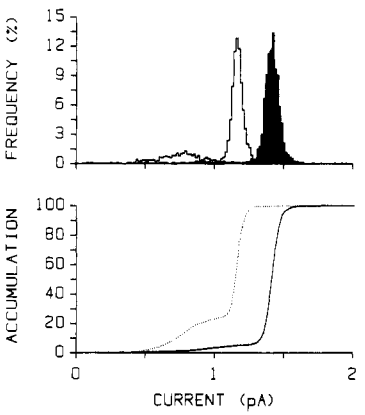

FIGURE 4: Current transition histograms and distribution functions of gramicidin A channels in DPhPC/decane membranes before and after addition of 8 μM Triton X-100. The curves in the lower half plot the accumulation of events in the histograms in the upper half. The "filled" histogram and solid distribution curve are based upon 1350 events from 4 consecutive measurements on the same day. The main distribution contains 1263 (96%) events where the average single-channel current was 1.40 ± 0.05 pA (mean ± SD). The average current in the individual measurements varied from 1.39 to 1.42 pA. The instrumental noise was estimated to be 0.05 pA. The "empty" histogram and the stippled distribution function are based upon 3182 events from 8 consecutive measurements after the addition of 8 μM Triton (Sigma). The main distribution contains 2417 (76%) events where the average single-channel current was 1.15 ± 0.04 pA. The average current in the individual measurements varied from 1.12 to 1.20 pA. The instrumental noise was estimated to be 0.02 pA. Experiment 3 of Table I.

each second—the average number of open channels was >1. In six determinations of the quantitative change in channel activity, the conductance increased between 7- and 80-fold; the average was 40-fold.

The detergent-induced increase in channel appearance rate could result from an increase in the gramicidin concentration in the membrane, by an increase in the rate constant for the monomer-to-dimer association, or from a combination of these mechanisms. In these experiments, gramicidin was added to the electrolyte, and it is possible that Triton increased the channel activity by promoting the membrane insertion of gramicidin monomers from the electrolyte solution. To address this question, membranes were formed from lipid/hydrocarbon solution to which gramicidin had been added at a peptide/lipid mole ratio of $\sim 10^{-7}$. Under these conditions, the gramicidin concentration in the membrane should be constant. No channels were observed during several minutes of small membrane current recording at 100-mV applied potential. The small signal conductance of the large membrane was then recorded before and after the addition of 8 μM Triton X-100. In four experiments, the large membrane conductance increased between 25- and 60-fold (with an average of 40-fold), and channels were seen in small membrane recordings in each experiment after Triton addition. The increased channel appearance rate can be explained solely by an increased association rate constant.

The increased channel stability was quantified in duration histograms (presented as survivor plots) for gramicidin channels in the absence and presence of Triton X-100 (Figure 3). For either situation, the distributions were well described by single-exponential decays for more than four time constants, and the increase in channel stability was quite reproducible. In nine experiments with 8 μM Triton (from three different sources), the average channel duration increased 5-fold (from 560 to 2850 ms) (see also Table I). We did not attempt to quantify the time course of the increase in channel stability. The complete effect was seen within 5 min of Triton addition.

In addition to these effects on channel "gating", Triton addition alters the rate of ion permeation through gramicidin

channels. At 8 μM Triton X-100, the magnitude of the most probable conductance decreases 15–20%. This effect is barely discernible in the current tracings in Figure 2 but is readily seen in current transition histograms. In Figure 4, for example, the average single-channel current transition for events in the main peak decreases from 1.40 to 1.15 pA upon Triton addition (see also Table I). The full conductance decrease was seen within 5 min of Triton addition.

Inspection of Figure 4 shows a further effect of Triton addition, which will be the focus of this paper, namely, an increased heterogeneity of the single-channel current transition amplitudes. This is already evident in the current tracings in Figure 2, where several minis are seen after Triton addition. The increase in mini channel frequency is most conveniently quantified by using the amplitude distribution function (Figure 4, bottom). In the absence of Triton (solid curve, "filled" histogram), there is a sharp peak that contains more than 94% of the current transitions as estimated from the "breaks" in the distribution function. In the presence of Triton (dotted curve, "empty" histogram), only ~76% of the current transitions fall within the main peak. Most of the remaining transitions have amplitudes that are less than those of the standard channels. Despite this increased heterogeneity, the variation in current transition amplitudes among the standard channels (as quantified by the standard deviation of the distribution in the main peak), is, if anything, less in the presence than in the absence of Triton. This results primarily from a decrease in the instrumental noise upon addition of Triton (see legend to Figure 4).

In the absence of Triton, the mini frequency in this laboratory is less than that reported by most other laboratories. We believe this difference cannot be ascribed to different sampling or analysis methods (Busath et al., 1987). In order to further substantiate this point, single-channel current activity was recorded on a digital tape recorder and analyzed at different filter frequencies (20, 50, 200 Hz; the duration of the records used to determine the amplitude of the current transitions was 100, 40, and 10 ms, respectively). Increasing the filter frequency decreased the apparent mini frequency from 4% at 50 Hz to 1% at 200 Hz for control conditions, and from 13% at 50 Hz to 5% at 200 Hz in the presence of Triton X-100. The apparent decrease in mini frequency results from a broadening of the main peak (the standard deviation increased from ±0.24 pA at 50 Hz to ±0.48 pA at 200 Hz in the absence of Triton, and from ±0.22 pA at 50 Hz to ±0.39 pA at 200 Hz in the presence of Triton), because shorter current records are used to determine the current transition amplitudes [cf. Figure 4D in Urry et al. (1984a)]. Mini channels with conductances that are close to the standard channel conductance are thus included into the broader standard channel population. In addition, mini channels with conductances that are less than 10% of standard channel conductance tend to get "lost" in the more noisy base line at the higher filter frequency. When the filter frequency was decreased to 20 Hz, the mini frequency was 7% under control conditions and 13% in the presence of Triton X-100. The increased mini frequency in the absence of Triton results from the inclusion of "minis" with *very* low conductances ($\simeq$1.0 pS). These are not detected when the filter frequency is increased. The similar mini frequency at 50 and 20 Hz in the presence of Triton suggests that these minute channels either are not significant in number in the presence of detergent or are not biased against as seriously due to the decreased base-line noise in the presence of detergent (vide supra). The prevalence of these channel types was estimated by inspecting current traces

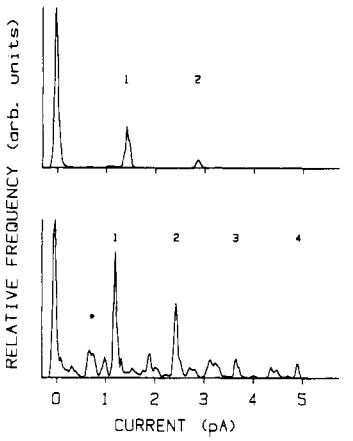

FIGURE 5: Current amplitude histograms in the absence and presence of Triton X-100. (Top) Histogram from a single measurement in the absence of Triton. The curve plots the relative amount of time that the membrane current was at a given value. This histogram includes information based on 470 s of continuous current recording (sampling frequency of 500 Hz) during which 340 current transitions were resolved. The numbers above the well-defined peaks represent the corresponding number of conducting channels at each peak. (Bottom) Histogram from a single measurement on the same day after the addition of 8 μM Triton X-100 (Pierce). This histogram includes information based on 728 s of continuous current recording during which 417 current transitions were resolved. There are well-defined peaks where 0–4 channels are conducting, as well as rather poorly defined peaks corresponding to the mini channel channel levels (e.g, the one marked by an asterisk). Both measurements are from experiment 7 of Table I.

by eye on several days. Under control conditions, 2–3% of all channels are of the very low conductance type; in the presence of Triton, only 1% of all channels are of this type. Hence, we believe they are mechanistically unrelated to the mini channels induced by detergent. [These very small channels are frequently observed in membranes which are "thick" (nonbilayer) by capacitance measurements, again suggesting that they occur by a different mechanism than the mini channels reported here.]

The Triton-induced increase in mini frequency was likewise not a result of the analysis procedures used. This can be illustrated in current amplitude histograms obtained in the absence or presence of Triton (Figure 5). In the absence of Triton (top) there are three well-defined peaks corresponding to zero, one, and two conducting channels, respectively. In the presence of Triton (bottom), there are five well-defined peaks, corresponding to zero, one, two, three, and four conducting channels, respectively. In addition, there are a number of rather broad peaks that were not seen in the absence of Triton. These peaks denote the existence of conductance variants, i.e., mini channels. It should, in particular, be noted that the "peak" denoted by an asterisk is at a position (relative to the "zero" and "one" channel peaks) that corresponds to that of the mini channels in Figure 4.

The Triton-induced increase in mini frequency occurs in two phases. There is an immediate (>2-fold) increase, which is complete within 10 min of Triton addition, followed by a slow and variable increase, where the mini frequency may increase an additional factor of 0.5 over the next several hours. This slow and time-dependent increase in mini frequency, taken together with a decreased membrane stability at higher detergent concentrations, precluded attempts to obtain dose–response curves. In contrast to the situation in the presence of Triton, in the absence of Triton there was no time-dependent trend in the mini frequency [see also Busath et al. (1987)].

Mini channels have a characteristic distribution in current transition histograms, which is particularly apparent in Figure

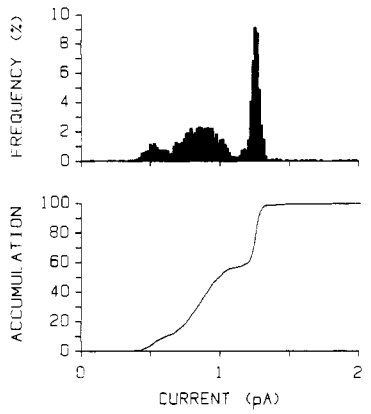

FIGURE 6: Current transition histogram and accumulation plot of gramicidin A channels in the presence of 8 μM Triton X-100 (Calbiochem). The figure is based upon 2956 events from 8 consecutive measurements. The main peak contains 1436 (49%) events with an average current of 1.24 ± 0.05 pA. The average amplitude of the standard channels varied from 1.21 to 1.26 pA among the individual membranes. There is an apparently bimodal distribution of the mini channel amplitudes. The mini channels in the lowest peak had current transitions ranging from 0.34 to 0.63 pA, with a mean of 0.52 ± 0.06 pA (N = 286). Channels in the middle peak ranged between 0.64 and 1.09 pA with a mean of 0.85 ± 0.11 pA (N = 1145). Experiment 2 of Table I.

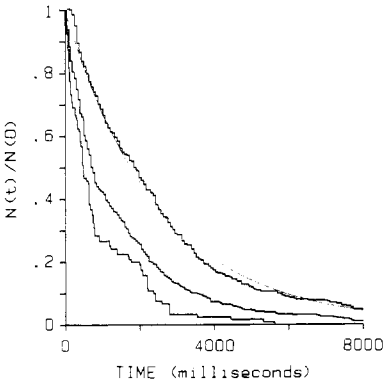

FIGURE 7: Normalized survivor plots of the three distributions of channels from the experiment used for Figure 6. Single-channel durations for each of the three peaks in the current transition histogram were analyzed and plotted as separate distributions. The distribution with the shortest average duration corresponds to the lowest peak of mini channels; the distribution of longest average duration corresponds to the standard channels (the curve represents a fit of a single-exponential decay to the results); the distribution of intermediate duration corresponds to the middle peak in the amplitude histogram. The distributions of the mini channels could not be fit by single exponentials. For the 306 standard channels, τ = 2550 ms [$N(0)$ = 310]; for the 382 "large" mini channels, $\tau \simeq$ 1720 ms; for the 120 channels in the "small" mini distribution, $\tau \simeq$ 1130 ms. Experiment 2 of Table I.

6 where we illustrate the current transition histogram and distribution function from the experiment with the largest Triton-induced mini frequency (Table I, experiment 2). In this case, the mini channels fall within two broad peaks. The duration distributions of the channels in the three peaks of the current transition histogram are shown in Figure 7. The standard channels have the longest average duration. A similar result was obtained in another experiment where there were sufficient numbers of mini channels to permit analysis of their duration distribution (data not shown). Among the mini channels, the population with the lowest conductance had the shortest average duration. The mini channel duration distributions could not be described by single exponentials, however, which suggests that there are multiple components in each peak, as is also indicated by the width of the "peaks" in the current transition histogram.

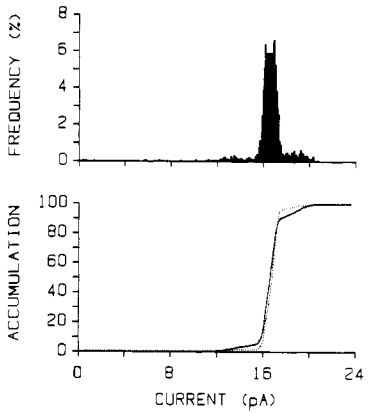

FIGURE 8: Distribution of gramicidin A channel transitions in 0.1 M HCl (pH 1.3) in the absence and presence of 8 μM Triton X-100. Only the current transition histogram in the presence of Triton is illustrated; the corresponding accumulation plot is given by the continuous curve. The stippled distribution curve is based upon 228 events from 2 measurements in the absence of Triton on the same day. The main peak for the control experiments contains 216 (95%) events where the average single channel current was 16.6 ± 0.44 pA, τ = 307 ms. The average current amplitudes in the individual measurements were 16.3 and 17.0 pA. Of the 12 (5%) nonstandard channels, 5 (2%) were minis, and 7 (3%) were maxis. The histogram and solid distribution function are based upon 2012 events from 6 consecutive measurements after the addition of 8 μM Triton (Calbiochem). The main peak contains 1721 (86%) events with an average amplitude of 16.5 ± 0.45 pA, τ = 653 ms. The average current in the individual measurements varied from 16.1 to 17.1 pA. Of the nonstandard transitions, 104 (5%) were minis, and 187 (9%) were maxis.

There is another, less quantifiable, aspect of the mini channels that is worth noting as it provides some insight into the mechanism of mini channel formation. On the basis of recordings (taken over many days) where no more than two or three channels conduct at any time, fewer than one channel per several thousand ever changed conductance level during its duration. Even in the experiment of Figure 6 where on average every other channel was a mini, no clear transitions between current levels in a single channel were observed. In five channels where such transitions were resolved, all began with a standard current amplitude and within 100 ms of their transition to another level either returned to the standard level (in three cases) or closed (in two cases). Considering the similar stability of mini and standard channels (see Figure 7), these brief flickers to nonstandard conductance levels do not appear to be mechanistically related to typical mini channels (see also Discussion).

To examine whether the Triton-induced increase in mini frequency varied with the type and concentration of the permeant alkali metal cation, measurements were also made in 0.1 M NaCl and in 1.0 and 0.1 M CsCl. The results were similar to those reported for 1.0 M NaCl (results not shown). Triton addition also induced an increased conductance heterogeneity when H^+ was the permeant ion, but in this case, there was a large increase in the frequency of channels that had conductances *above* the main peak (i.e., maxies) as well as an increased mini frequency (Figure 8). This result is consistent with the finding of Busath and Szabo (1988b) on spontaneously occurring conductance variants.

Triton X-100 in the Absence of Gramicidins. At sufficiently high concentrations ($\gtrsim$150 μM), Triton X-100 can induce channellike activity in planar bilayers (Schleiper & De Robertis, 1977; Benz & Hancock, 1981). At the low concentrations used in this study, however, Triton addition in the absence of gramicidin did not induce discrete current steps (channels) in planar bilayers (results not shown). After ad-

Table II: Gramicidins B and C with Triton X-100

expt	peptide	I (pA) ± SD	N^a	% minis	τ (ms)	N^b
1	gramicidin B[c]	2.00 ± 0.08	1423	3.7	1680	666
	+8 μM Triton[d]	1.67 ± 0.06	3373	15.8	8270	325
2	gramicidin B[c]	1.88 ± 0.10	2894	4.7	1830	1129
	+8 μM Triton[e]	1.61 ± 0.05	1284	24.3	(8670)	(89)
3	gramicidin C	1.25 ± 0.06	861	4.3	370	710
	+8 μM Triton[d]	1.04 ± 0.04	1690	6.0	1710	454
	+16 μM Triton[d]	0.97 ± 0.04	1433	10.3	3900	221
4	gramicidin C	1.24 ± 0.07	804	6.1	470	360
	+8 μM Triton[e]	1.02 ± 0.03	1773	13.5	1590	625
	+16 μM Triton[e]	0.96 ± 0.03	887	15.7	(3760)	(60)

[a] Number of events in the aggregate current transition histogram. [b] Number of channels in the aggregate channel duration histogram. Parentheses around survivor plots with fewer than 100 channels. [c] 200-mV applied potential. [d] Triton X-100 from Calbiochem. [e] Triton X-100 from Sigma.

Table III: Gramicidin A with Structurally Distinct Detergents

expt	detergent	I (pA) ± SD	N^a	% minis	τ (ms)	N^b
1	–	1.43 ± 0.04	1757	9.7	540	594
	8 μM reduced Triton	1.18 ± 0.02	2098	25.2	2830	452
2	–	1.36 ± 0.03	335	5.7	580	160
	8 μM Genapol X-100	1.24 ± 0.07	1906	13.1	2300	426
3	–	1.50 ± 0.04	132	4.6	(540)	(67)
	8 μM Genapol X-100	1.23 ± 0.05	1406	16.2	3120	147
4	–	1.36 ± 0.05	2196	3.6	500	1074
	0.9 μM octyl β-glucoside	1.31 ± 0.05	3895	12.6	2650	452
5	–	1.34 ± 0.05	1393	2.4	530	702
	2 μM lysolecithin	1.13 ± 0.04	1058	9.3	1620	266
6	–	1.40 ± 0.04	727	7.8	620	387
	56 μM Zwittergent 3-12	1.34 ± 0.07	1760	9.7	1000	432
7	–	1.37 ± 0.04	102	5.9	(860)	(46)
	112 μM Zwittergent 3-12	1.20 ± 0.03	1242	7.5	1940	187

[a] Number of events in the aggregate current transition histogram. [b] Number of channels in the aggregate channel duration histogram. Parentheses around survivor plots with fewer than 100 channels.

dition of 8 μM Triton X-100, the background conductance of the large membranes could increase up to 2-fold. At 200 mV, the trace was quiet for several minutes, with no discrete membrane current changes.

Reactive Contaminants Cannot Explain the Increased Mini Frequency. Detergents may be contaminated by reactive compounds, such as peroxides and other oxidants, that can modify proteins covalently (Ashani & Catravas, 1980; Chang & Bock, 1980). To determine whether the increased mini frequency was a primary result of Triton itself, or alternatively was due to impurities in the Triton, we tested samples of Triton from different sources and of different nominal purities. The results are summarized in Table I. In all experiments, the mini frequency increased following detergent addition, though the variation in the increase was considerable. Even for Triton from the same source, the increase in mini frequency could vary more than 3-fold from day to day, and among membranes on a given day. Despite this large day-to-day variation in the Triton-induced mini frequency, it appears that one sample of Triton (Pierce) induces fewer minis than do the others. In fact, the increase was *very* small in two experiments with Pierce Triton (Table I, experiments 5 and 6). We believe, however, that this should be ascribed to the quite high mini channel frequency in the control measurements before Triton addition.

That different Triton preparations seem to differ in their ability to induce mini channels could suggest that the increased mini frequency resulted from covalent modification of the gramicidins by reactive contaminants. To examine this question further, small aliquots of the stock solutions of gramicidin A or B were incubated in 250 μM Triton X-100 for 24 h before the peptides were added to the electrolyte solution. The final Triton X-100 concentration was less than 1 μM. The mini frequencies ranged from 2.1% to 8.7%, similar to those results obtained with gramicidin that had not been incubated with Triton (control results in Table I).

Experiments with Other Gramicidins. The Triton-induced increase in mini frequency is not specific for gramicidin A channels, as similar results were obtained with channels formed by gramicidins B and C. The results are summarized in Table II. There are again large variations in the Triton-induced increase in mini frequency, but the general effect is the same for channels formed by each of the gramicidins.

Effect of Other Detergents and Membrane Modifiers.[2] The ability of Triton X-100 to increase the mini channel frequency appears to be a quite general detergent effect, as comparable increases were induced by many other detergents. Triton X-100 has a long hydrophilic polyoxyethylene chain and a short hydrophobic tail that contains a benzene ring. A possible mechanism by which Triton could increase the mini channel frequency is thus through interactions between the aromatic ring and the indoles of the tryptophanyl residues near the channel entrances. This could alter the average orientation of the indoles, which again could alter the channel conductance. To test this possibility, we examined the effect of reduced Triton X-100, where the aromatic ring has been hydrogenated to a cyclohexane. The effects of this detergent were indistinguishable from those of (nonreduced) Triton X-100 (Table III). Likewise, Genapol X-100, which has a linear 12-carbon hydrocarbon tail and a hydrophilic group similar to Triton, produced the same changes in channel behavior as the Tritons (Table III). The mini channel inducing ability of Triton X-100 cannot, therefore, be critically dependent on

[2] In these experiments, the detergent concentrations were adjusted relative to the critical micellar concentrations (CMC) for each detergent. The detergents were tested at ~0.01 × CMC, which should ensure that the membrane concentrations should be comparable for all detergents (see Discussion).

Table IV: Gramicidin A in Different Lipids, with and without Triton X-100

expt	lipid	I (pA) ± SD	N^a	% minis	τ (ms)	N^b
1	DOPC	1.41 ± 0.04	2323	4.1	530	1181
	Triton[c]	1.32 ± 0.03	2767	12.8	1740	588
2	DOPS	2.01 ± 0.05	751	10.0	560	326
	Triton[c]	1.84 ± 0.05	1133	26.1	(1550)	(35)
3	GMO	2.58 ± 0.06	1457	8.1	480	539
	Triton[c]	2.21 ± 0.06	1525	11.6	570	300
4	GMO	2.58 ± 0.07	1560	8.3	430	372
	Triton[c]	2.18 ± 0.05	830	21.1	620	254

[a] Number of events in the aggregate current transition histogram. [b] Number of channels in the aggregate channel duration histogram. Parentheses around survivor plots with fewer than 100 channels. [c] 8 μM Triton X-100 from Calbiochem.

the nature of its nonpolar constituent.

To test whether the polar polyoxyethylene moiety was essential, a number of other detergents were examined. Octyl β-glucoside is nonionic with a carbohydrate polar moiety; addition of octyl β-glucoside produced increases in mini channel frequency, channel stability, and channel activity that were similar to those of Triton, but there was no effect on the standard channel conductance (Table III). Monopalmitoylphosphatidylcholine (lysolecithin) is zwitterionic, with a glycerophosphocholine polar moiety; it induces changes in gramicidin channel behavior that are comparable to those induced by the Triton-like detergents. Another zwitterionic detergent, Zwittergent, where the polar moiety is a [(dimethylamino)-propyl]sulfonic acid, induced less dramatic increases in channel activity and duration, decreased the standard channel conductance only slightly, and had no clear effect on the mini channel frequency.

Mini Channels in Different Lipids. Results from other laboratories suggest that the intrinsic mini frequency may vary with the bilayer composition [Bamberg et al., 1976; but see Busath and Szabo (1988a)]. This has been interpreted to mean that the ability of the side chains in gramicidin channels to undergo discrete and stable configurational changes, that may lead to altered single-channel conductances, may differ among channels in bilayers of different composition [Läuger, as quoted in Edholm et al. (1984)]. Dioleoylphosphatidylcholine (DOPC), in particular, has been implicated as promoting the formation of mini channels, but we were unable to substantiate this suggestion. For gramicidin A channels in DOPC membranes, we observed mini frequencies that were similar to those found in DPhPC bilayers (Table IV). Triton addition generally had the same effect on gramicidin channels in DOPC bilayers as in DPhPC bilayers.

We also investigated the spontaneous and detergent-induced mini channels in dioleoylphosphatidylserine (DOPS) and glyceromonoolein (GMO) membranes and found the same general behavior of gramicidin (mini) channels in these membranes (Table IV). Note also that Triton did not induce the same increase in channel duration in GMO membranes as in the phospholipid membranes. The relative change in the single-channel current transitions, however, was comparable to that seen in phospholipid membranes.

Possible Sources of Detergent Contamination. The large interlaboratory variation in mini channel frequency is most likely a consequence of differences in the way different laboratories handle their gramicidin, lipid, and electrolyte solutions (Busath et al., 1987). In particular, it is possible that the variations result from inadvertant detergent contamination. We investigated two possible sources of detergent contamination.

First, the electrolyte solutions are sometimes filtered, and wetting agents are used to pretreat filters with pore diameters ~0.22 μm (based on suppliers' information). To examine whether such filters can leach detergents in quantities sufficient to alter the single-channel behavior of gramicidin, electrolyte solutions were filtered before use. This made the membranes less stable and more difficult to work with, but there were no quantitative differences between channel behavior in the filtered versus the unfiltered electrolyte: in two experiments with filtered electrolyte, the average conductance was 13.6 pS, and the mini frequency was 5.3% (4.7% and 5.4%).

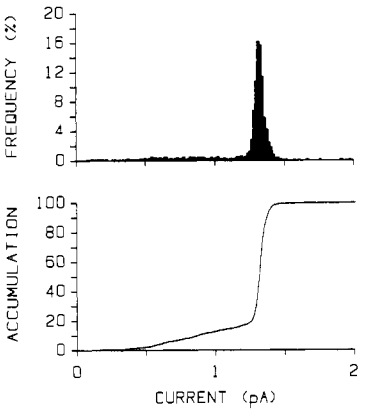

FIGURE 9: Current transition histogram of gramicidin A in electrolyte prepared in a detergent-washed rather than an acid-washed flask. The flask was borrowed from a laboratory that washes its glassware with detergent. The histogram and distribution curve are based upon 1979 events from 10 consecutive measurements on the same day. The main peak contains 1642 (83%) events where the average single-channel current was 1.31 ± 0.05 pA. The instrumental noise was estimated to be 0.03 pA. The average current in the individual measurements varied between 1.29 and 1.33 pA. For the channels in the main peak, τ = 750 ms [$N(0)$ = 760]. In an identical experiment including 1548 events from 10 consecutive measurements, the main peak contained 1334 (86%) events where the average single-channel current was 1.36 ± 0.04 pA. [The average currents varied between 1.32 and 1.42 pA, τ = 560 ms [$N(0)$ = 620].]

Second, there may be residual detergent left in glassware that is cleaned by using standard laboratory detergents. To test this, we borrowed detergent-washed flasks from another laboratory to make up the electrolyte solutions (as all of our glassware is washed in dichromate–sulfuric acid). In this case, the channels had a different behavior from that usually observed in the absence of added detergent (Figure 9). In two experiments with electrolyte prepared in detergent-washed 50-mL volumetric flasks, the mini frequency was increased compared with the usual controls (Figure 9; cf. Table I), consistent with the notion that residual detergents can leach from the glassware in sufficient quantities to alter the channel behavior.

Discussion

In this paper, we show that the molecular behavior of gramicidin channels can be markedly altered by a variety of detergents and that these detergent-induced alterations occur at detergent concentrations much less than the critical micellar

concentrations (CMC). Detergents alter all major aspects of channel function: the channel appearance rate is increased; the channel disappearance rate is decreased; the single-channel conductance is (usually) decreased; and there is an increased conformational heterogeneity, as reflected in the increased mini channel frequency.

That detergent addition induces an increase in mini channel frequency shows that the mini channel phenomenon cannot be an intrinsic channel characteristic, or more precisely that most, if not all, mini channel appearances result from interactions among channel-forming peptides, the host bilayer, and adsorbed detergents. This conclusion extends that reached by Busath et al. (1987), namely, that mini channels occur as a result of environmental modulations of the channels. Here we provide a specific example of environmental perturbations that alter channel behavior.

We first show that detergent-induced mini channels are similar to, if not identical with, the spontaneously occuring mini channels seen in our and other laboratories. Next, we address the issue of whether detergents act as catalysts or as cofactors in the induction of mini channels. We then discuss possible conformational changes that could lead to mini channels and how membrane-adsorbed detergent might induce such conformational changes. Finally, we briefly address the other effects of detergent on gramicidin behavior as well as the possible implications of these results for the study of membrane proteins in general.

Detergent-Induced vs Spontaneous Mini Channels. Detergent-induced mini channels appear to be similar to the mini channels that are observed in the absence of detergents: *First*, the detergents themselves do not form channels; the detergent-induced mini channels are gramicidin channels. *Second*, mini channels do not result from covalent modification of the channel-forming peptides.[3] *Third*, Triton-induced mini channels (Figures 4, 5, and 8) have approximately the distributions that have been reported for spontaneously appearing mini channels [Figure 6 in Urry et al. (1984c), Figure 5 in Busath et al. (1987), and Figure 4 in Busath and Szabo (1988a)]. We note, in particular, that mini channel distributions at high mini frequencies tend to become approximately bimodal (Figure 5), a pattern that also can be seen in results from other laboratories. *Fourth*, with H^+ as the permeant ion, Triton induces channels that have *increased* conductances (Figure 8), similar to what was reported for the spontaneously occurring conductance variants by Busath and Szabo (1988b). *Fifth*, while detergent addition increases the overall channel stability, in the presence of Triton the average duration of the mini channels is less than that of the standard channels, in agreement with the findings of Busath and Szabo (1988a) for spontaneously appearing minis. *Finally*, (*sixth*) on a qualitative level, we find that both mini channels and standard channels were stable, with no convincing evidence for transitions between different current levels while a channel is conducting, in agreement with the reports for spontaneously occurring mini channels (Urry et al., 1981).[4] None of the individual arguments are by themselves conclusive, but together they strongly suggest that detergent-induced mini channels are similar to spontaneously appearing mini channels.

Detergent–Peptide Interactions. To characterize the detergent–peptide interactions responsible for mini channel formation, it is necessary to have estimates for the detergent concentration in the membranes. These were obtained by using an extension of the model of Nichols and Pagano (1981) [see also Bullock and Cohen (1986)]. The detergents are present at concentrations much less than the critical micellar concentrations, and it is assumed that bilayer-adsorbed detergent monomers (D_b) are in free exchange with detergent monomers in the aqueous solutions (D_a):

$$D_a \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} D_b$$

where k_1 and k_{-1} denote the rate constants for detergent adsorption and desorption, respectively. The exchange rate is given by [e.g., see Bullock and Cohen (1986)]

$$d[D]_b/dt = -k_{-1}[D]_b + k_1[D]_a([D]_b d + [L]_b l) \quad (1)$$

where $[D]_b$ and $[L]_b$ denote the membrane surface concentrations of detergent and lipid in each half of the bilayer, respectively, $[D]_a$ is the aqueous detergent concentration, and d and l are the molar areas of detergent and lipid, respectively. At equilibrium, $d[D]_b/dt = 0$, and

$$[D]_b/([D]_b d + [L]_b l) = [D]_a K_{ads} \quad (2)$$

where K_{ads} ($=k_1/k_{-1}$) is the adsorption coefficient for the detergent. The mole fraction of D in the membrane, x_D, is given by $[D]_b/([D]_b + [L]_b)$, when $[D]_b \ll [L]_b$ (or, more precisely, when $[D]_b d \ll [L]_b l$), $x_D \simeq [D]_b/[L]_b$, and eq 2 can be rewritten as $x_D = [D]_a l K_{ads}$. If detergents interact with membrane lipids as they do with each other in micelles, $1/K_{ads}d$ should be equal to the CMC (Bullock & Cohen, 1986), and the mole fraction of detergent in the membrane should be related to the aqueous detergent concentration by

$$x_D = ([D]_a/\mathrm{CMC})(l/d) \quad (3)$$

On the basis of inspection of CPK models, l/d varies between 1 and 3. In our experiment, the detergent concentrations varied between 0.025(CMC) and 0.1(CMC) (for Triton X-100 and lysophosphatidylcholine, respectively), and x_D is predicted

[3] It is in this respect important to distinguish conductance heterogeneities that result from conformational variants on the $\beta^{6.3}$-helical dimer scheme (mini channels) from those resulting from channel formation involving convalently altered, e.g., photolyzed (Busath & Waldbillig, 1983), gramicidins. We only consider the former case, but we cannot exclude that the residual (control) mini frequency seen in the absence of detergents (in part) could reflect the presence of such covalently modified gramicidins. Nor can we exclude that mini channels that are seen in the nominal absence of detergent result from trace contaminants in the lipid.

[4] Transitions between standard and "mini" current levels have been observed and compared to stable mini channels (Busath & Szabo, 1981, 1988a). Most of the current traces in these papers show transitions between the standard level and a current level that is *not* typical of the mini channel in the accompanying current transition histograms. This level is comparable to the very small mini channels that we believe are mechanistically unrelated to the majority of mini channels because their frequency is not changed by detergent. Hence, these transitions do not constitute evidence for easy interconversion between the standard and mini channel conformations. Other efforts to estimate the frequency of conductance-state interconversions (Urry et al., 1984a) are also problematic. These authors used the following criterion to detect conductance interconversions: if the magnitude of the current transition of the channel appearance differed from that at the disappearance by an amount that was larger than 1 standard deviation of the standard channel peak, it was declared to be a state change. However, the width of the transition histograms is primarily instrumental (cf, legend to Figure 4). On a statistical basis, therefore, only 67% of any two "identical" transitions will have amplitudes that differ by less than 1 standard deviation. Hence, this procedure would lead to an estimate of 0.33 for the probability that any channel did have an interstate transition. The reported probability was 0.35!

to be ~0.05 for Triton and octyl β-glucoside, and ~0.1 for lysophosphatidylcholine.[5]

At the rather low detergent concentrations used here, the surface density of detergent molecules in the bilayers is ~1 per 10 nm². A membrane-spanning $\beta^{6.3}$-helical gramicidin dimer is surrounded by an annulus of ~10 phospholipid molecules (in each half of the bilayer) that are in immediate contact with the peptide. The time-averaged number of detergent molecules in each annulus is thus on the order of 1! In any case, given diffusion coefficients of lipids and proteins in hydrocarbon-containing bilayers of 10^{-7} cm²/s (Fahey et al., 1977), the encounter frequency between a conducting gramicidin dimer and the detergent molecules is $>10^7$ s^{-1}, but both standard and mini channels are stable, over several seconds, with no discrete conductance changes (from the standard level to a well-defined mini level, or vice versa). The mere contact between a channel and a detergent molecule is therefore not sufficient to induce the conformational change that is associated with a mini channel (but *could* well induce the decrease in standard channel conductance; see below). Rather, it seems that the conformational change that determines conductance level must take place before the channel conducts, i.e., during peptide folding and insertion into the membrane.

There are thus two possible mechanisms by which detergents could induce mini channels: a *direct* mechanism, in which the detergent acts as a cofactor in a noncovalent, permanent, association between peptide and detergent during channel folding and insertion, which alters (however subtly) the channel conformation; an *indirect* mechanism, in which the detergent acts as a catalyst, by altering some characteristic of the host bilayer, which leads to a decrease of the energy barrier for interconversion among different conformational substates during the folding and insertion step(s). These two mechanisms are not mutually exclusive, but in the indirect (catalytic) mechanism, the detergent need not remain associated with the channel once it has formed.

The catalytic mechanism is supported by the observation that detergents increase the general gramicidin activity in bilayers, by promoting peptide folding/insertion or monomer association per se. In the absence of detergent, however, the mini channel frequency is stable over hours, suggesting that mini and standard channels (on our time scale) are in equilibrium. If equilibrium indeed is attained, the catalytic mechanism cannot be the dominant mechanism by which mini channels are produced, since detergents increase the (absolute and relative) concentration of mini channels (Figure 4), which implies that the equilibrium constant for mini channel formation has been changed. Mini channel formation thus seems to involve a direct mechanism; i.e., peptides and detergents remain associated for the duration of a channel appearance.

We can obtain an estimate for the dissociation constant, K, of this putative gramicidin–detergent complex, because it seems that only one monomer in the dimer needs to be altered in a mini channel (Busath & Szabo, 1988b). Accordingly, if the association between normal and altered monomers is energetically indistinguishable from the association between two normal monomers, the mini channel frequency, f_m, should be a function of the mole fraction of the altered monomers

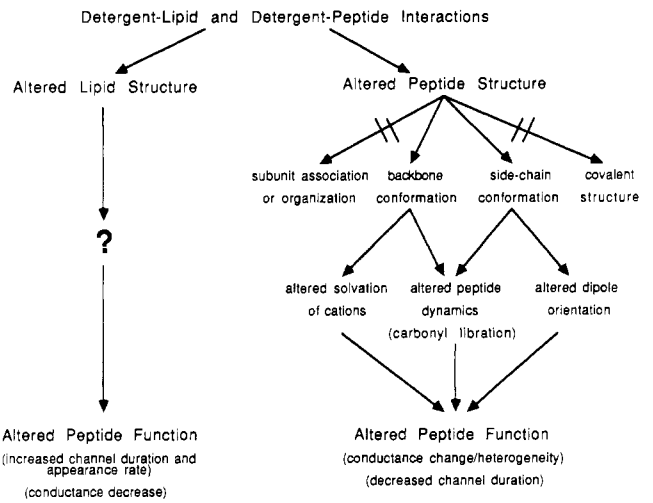

FIGURE 10: Flow chart to summarize how detergent might interact with lipids and proteins to alter the behavior of gramicidin channels. The changes in channel activity, duration, and standard channel conductance appear to result from detergent–lipid interactions, which change the physical properties of the bilayer, and probably also from nonspecific (rapid) detergent–peptide interactions. Mini channels result from stably altered peptide conformations that most likely result from rather specific and direct peptide–detergent interactions. Some of the possible ways that a change in peptide conformation may result in changes in channel function are indicated. Changes in the covalent structure of the monomers or in subunit structure cannot account for the mini channels (see text). This leaves alterations in backbone structure and reorientation of the amino acid side chains as likely candidates to account for the properties of mini channels. These structural changes could alter the channel's permeability to cations: by altering the electrostatic barrier to ion movement; by altering the carbonyl librations; by altering the ability of the channel entrance to solvate ions and H_2O; or from a combination of these effects.

(relative to all gramicidin monomers), x_m', as [e.g., see Mazet et al. (1984)] $f_m = 2x_m' - x_m'^2$. In our experiments, f_m is ~0.2, so x_m' is ~0.1, but $K = x_s x_D / x_m$, where x_s and x_m denote the mole fractions of standard and altered monomers in the membrane, respectively. With x_D in the range of 0.05–0.10 and $x_s/x_m \simeq 10$, we estimate that K is ~1. There thus appears to be little energetic preference for peptide–detergent vs peptide–lipid interactions.

On the Structure of Gramicidin Mini Channels. The altered conductances of mini channels imply that their conformations are altered. Possible mechanisms by which conformational changes could produce altered conductance states are summarized in Figure 10. Standard gramicidin channels are NH_2-terminal-to-NH_2-terminal dimers of $\beta^{6.3}$-helices, as originally proposed by Urry (1971). It appears that mini channels represent comparatively minor conformational variations of the standard channel conformation (Busath & Szabo, 1988a). Detergent-induced mini channels do not, for example, result from covalent modification of the channel-forming peptides. In addition, the broad distributions of mini channel conductances, taken together with the similar durations of standard and mini channels, tend to exclude simple mechanisms that involve major structural changes: a different helix sense; a different number of residues per turn; formation of membrane-spanning intertwined dimers; disruptions at the dimer join; or formation of head-to-tail or tail-to-tail dimers. The broad distribution of mini channel conductances and the presence of both mini and maxi channels with H^+ as the permeant ion suggest further that more than one mechanism should be invoked to account for the altered conductance of gramicidin mini channels. Three general mechanisms have been proposed (Busath & Szabo, 1981): an external (dipolar or charged) factor could bind close to the channel entrance,

[5] These estimates may be too high. The adsorption of octyl β-glucoside and Triton X-100 to phospholipid vesicles has been determined directly (Jackson et al., 1982; Goni et al., 1986). The adsorption of octyl β-glucoside is quite well predicted by eq 3, but the adsorption of Triton X-100 is about 10-fold less than predicted.

thereby altering the electrostatic contribution to the energy profile and thus the conductance; there might be (static) defects in the peptide backbone, such that ion solvation by the carbonyl oxygens was impaired; or different rotameric states of the large tryptophanyl and/or leucyl residues could be associated with different conductance levels. We have shown that mini channels indeed result from the association of an external factor, a detergent molecule, with the channel-forming peptides. It is unlikely, however, that the conductance changes result from simple electrostatic potential changes, as this would not explain the induction of maxi channels with H^+ as the permeant ion, nor the induction of similar mini channels by detergents with electrostatically quite dissimilar head groups.

Alterations in side chain orientations could alter channel conductances directly and indirectly. A direct effect could result from altered electrostatic interactions between permeating ions and the side chain dipole [e.g., see Mazet et al. (1984) and Koeppe et al. (1989); see also Pullman (1987)]. This seems unlikely, however, because replacement of a polar tryptophan residue with a nonpolar phenylalanine in gramicidin B has no effect on either the detergent-induced mini frequency or the mini frequency in the absence of detergent (Table II). Indirectly, the side chain orientations could alter the conductance by altering the dynamics of the peptide groups that line the channel wall and thus the ability of the carbonyl oxygens to librate into the channel lumen to form hydrogen-bonded contacts with H_2O or solvate permeating cations (Urry et al., 1981; Venkatachalm & Urry, 1983). Indirect effects involving alterations in ion solvation because of altered peptide libration are unlikely to be dominant, however, since a Na^+ mini can be a H^+ maxi (Busath & Szabo, 1988b), and carbonyl librations would presumably have similar roles in solvating an alkali metal cation or H_3O^+. It is also unclear whether different rotameric states would be sufficiently stable to account for the rarity of spontaneous transitions between different conductance states of a single channel.

Attempts to obtain experimental support for the importance of peptide libration in the genesis of mini channels have not been conclusive. Two different gramicidin analogues (des-[D-Val^6-L-Val^7]- and [Ala^7]gramicidin A) have the Trp^{13}–Val^7 contact replaced by a Trp–Ala contact, which should allow for greater conformational freedom of the tryptophanyls in channels formed by the analogues (Urry et al., 1984b; Prasad et al., 1986). Channels formed by des[D-Val^6-L-Val^7]gramicidin A did exhibit increased single-channel conductance heterogeneity (Urry et al., 1984b), but the apparent heterogeneity was *decreased* in [Ala^7]gramicidin A channels (Prasad et al., 1986). In light of the results reported here, these findings may have resulted from environmental differences between the experiments.

Alterations in backbone structure could, statically, alter ion–peptide and H_2O–peptide interactions and, dynamically, alter ion and water mobility in the channel because of altered peptide librations. Mechanisms that primarily invoke altered libration are unlikely to be dominant for reasons mentioned above. A structural change that alters the ion solvating ability of the channel entrance could account for many of the results. As a cation moves through a gramicidin channel, the ion must dehydrate (shed its solvation shell) as it enters the channel and rehydrate as it exits [e.g., see Andersen and Procopio (1980)]. The overall rate constant for either step should be affected by the geometry of the peptide groups at the COOH termini. Altered H_2O mobility could change the rate of ion permeation because metal cations and H_2O move in a single file (Levitt et al., 1978; Rosenberg & Finkelstein, 1978) and the rate of ion movement through the channel interior is determined by the overall H_2O mobility (Andersen & Procopio, 1980; Finkelstein & Andersen, 1981). It should, in particular, be noted that the H_2O mobility could decrease if H_2O molecules bind more tightly in the channel.

Protons, however, seem to jump along a chain of water molecules that fill the pore (Hladky & Haydon, 1972), which means that protons need not go through dehydration and rehydration at the channel entrances. A statically altered ion or H_2O solvating ability of the channel entrance need not, therefore, decrease the channel's proton permeability (Busath & Szabo, 1988b). It is, in fact, conceivable that the channel's proton permeability could be increased due to changes in water structure at the channel entrance, which increase proton mobility while decreasing alkali metal cation mobility [proton mobility is, for example, higher in ice than in water (Eigen & Maeyer, 1958; Conway & Bockris, 1958)]. It is therefore conceivable that the same structural change could lead to a decrease in the alkali metal cation permeability and an increase in the proton permeability.

The mini channel frequency increases with increasing temperature (Busath et al., 1987; Urry et al., 1984a). This is consistent with the notion that mini channels primarily arise because the backbone structure is loosened, as interactions that stabilize the backbone should weaken with increasing temperature. Amino acid side chains, on the other hand, would be expected to rotate more freely, making statically altered side chain orientations less likely.

Structural studies of gramicidin together with the properties of detergents as surface-active agents also provide clues as to how detergents might alter the structure at the channel entrance. Detergents disrupt protein–lipid interactions, and they denature both integral membrane proteins and soluble proteins (Helenius & Simons, 1975). Generally, detergents seem to "worm their way" into the proteins and destabilize the weak hydrophobic interactions that help to hold the protein in its native conformation. It is therefore not surprising that minute concentrations of detergent can have significant effects on the function of transmembrane channels. In this context, it may be useful to think of the gramicidin mini channel as a partially denatured structure. Specifically, the COOH termini form the channel entrances at the membrane/solution interfaces. The β-helical conformation of the entrances appears to be stabilized by specific interactions between the phospholipid carbonyls and the COOH termini (Wallace, 1986). Detergents could disrupt these interactions and thus alter the structure at the COOH terminus. The short des[D-Val^6-L-Val^7]gramicidin A channels should be particularly susceptible to such disruptions because of the greater strain imposed on the backbone of the shorter channel when it deforms the bilayer to form a membrane-spanning dimer. This could account for the large conductance heterogeneity of these channels (Urry et al., 1984b).

Zwittergent was the only detergent tested that did not clearly induce mini channels at concentrations up to 0.1- (CMC). Structurally, Zwittergent differs from the other detergents in that it should be less able to form hydrogen bonds at its polar head group. Perhaps then, in the mini channel, hydrogen bonds that normally form between different segments of the peptide backbone and/or between the backbone and the lipid head groups are replaced by interactions with an adsorbed detergent, thereby altering the backbone structure and thus the ion permeation path. Zwittergent had surprisingly little effect on other aspects of gramicidin channel behavior, however, and it is possible that Zwittergent does not adsorb as

readily as the other detergents.

Effects on Standard Channels. Detergents not only increase the mini channel frequency but also alter the "standard" channels in the major peak. These changes are of interest as examples of environmental modulation of channel function, but we will only discuss them briefly here. The increased channel appearance rate and stability are general detergent effects, as they were produced by all the detergents tested. Other changes in standard channel behavior may be more complex. Triton, for example, decreases gramicidin single-channel conductance comparably in all bilayers, irrespective of lipid composition, while its effect on channel duration depends on the membrane lipid composition. Thus, the change in gramicidin duration appears to result from detergent-induced changes in lipid–peptide interactions rather than from detergent–peptide interactions per se. The effect on channel activity appears to be similar to the octyl β-glucoside induced increase in colicin E1 incorporation into planar bilayers (Bullock & Cohen, 1986), and the facilitation of integral membrane protein incorporation into preformed phospholipid vesicles by cholate, myristolate, and cholesterol (Scotto & Zakim, 1985, 1986). These changes in gramicidin channel behavior should probably be interpreted following Huang (1986), i.e., in terms of changes in membrane characteristics that determine gramicidin channel duration (compression, surface tension, and splay). A detailed discussion is outside the scope of the present study.

The standard channel conductance changes result from more specific detergent–peptide interactions, but they do not appear to be related to the formation of mini channels. At detergent concentrations that induce comparable increases in mini frequency, Triton X-100, reduced Triton, Genapol X-100, and lysophosphatidylcholine decrease the standard channel conductance, while octyl β-glucoside has no effect. Zwittergent 3-12 decreases the conductance with no clear effect on the mini frequency. The conductance decreases induced by Triton X-100, reduced Triton, and Genapol X-100 are somehow related to their polar polyoxyethylene head groups, but it is not yet clear how these changes are brought about.

Implications. Many detergents, including Triton X-100 and octyl β-glucoside, alter the activity of detergent-solubilized enzymes [e.g., see Womack et al. (1983)], but these studies were usually done at detergent concentrations 3–4 orders of magnitude higher than those used here. These detergent effects have led to the general practice of dialyzing the detergents out of purified enzyme preparations. Even after careful dialysis, however, there is residual detergent present (Jackson et al., 1982), in concentrations that may be of the same order of magnitude as those that alter the properties of gramicidin channels. The ability of detergents, at low concentration, to alter the properties of gramicidin channels highlights the sensitivity of this membrane-spanning channel to changes in the environment, particularly to surface-active contaminants such as detergents that can perturb a normally quite ordered membrane structure. We also note that the nicotinic acetylcholine activated channel is converted to a completely desensitized state in the presence of Triton X-100, with a half-maximal effect at 16 μM (Kasai et al., 1970). The sensitivity of the mini frequency to the way in which glassware is washed should also change the way in which we view the gramicidin mini channel. As environmentally induced phenomena, however, the mini channels are particularly interesting, because they provide information about the role of the membrane environment in stabilizing gramicidin channels. In addition, mini channels may eventually help to elucidate the events that occur at the channel entrances during ion association and dissociation.

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Bis(sulfo-*N*-succinimidyl) Doxyl-2-spiro-5′-azelate: Synthesis, Characterization, and Reaction with the Anion-Exchange Channel in Intact Human Erythrocytes†

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ABSTRACT: We have synthesized and characterized bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate (BSSDA), a membrane-impermeant bifunctional spin-labeling reagent. BSSDA is a nine carbon backbone homologue of bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate [BSSDP; Beth et al. (1986) *Biochemistry 25*, 3824–3832]. Due to its longer backbone, BSSDA can span longer distances between reactive groups on a protein than can BSSDP. However, the purpose of the bifunctional design of these reagents is to provide a tight motional coupling of the spin labels to the surface of a target protein. To test whether the longer backbone of BSSDA results in a greater local flexibility and thereby undermines the effects of bidentate attachment, we have labeled with BSSDA anion-exchange channels of *intact* human erythrocytes at the same site as we have previously labeled them with BSSDP. Linear and saturation-transfer EPR spectra of BSSDA-labeled anion-exchange channels in intact cells closely approximate the corresponding spectra from BSSDP-labeled channels. Thus, the longer backbone of BSSDA relative to BSSDP does not give rise to significant local flexibility, even when BSSDA is bound to a site that can be spanned by the shorter reagent.

Biophysical techniques including EPR[1] spectroscopy have been utilized to characterize the dynamic properties of membrane proteins in a number of systems over the past decade. In order to carry out EPR measurements, it is ordinarily necessary to introduce a spin-label reporter group into the protein of interest, since most proteins lack endogenous paramagnetic centers. If the experimentalist is interested in the rotational diffusion coefficient of a membrane protein, a parameter that can provide insight into such diverse properties as state of oligomerization, interactions with other membrane proteins, and inherent flexibility, it is necessary to introduce a spin-label probe that will accurately monitor the motion of the protein to which it is covalently attached or noncovalently bound. Thus, the approach to be followed is to design a spin label that will exhibit limited independent motion relative to

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[1] Abbreviations: BSSDA, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-5′-azelate; BSSDP, bis(sulfo-*N*-succinimidyl) doxyl-2-spiro-4′-pimelate; BS^3, bis(sulfo-*N*-succinimidyl) suberate; $NaDodSO_4$, sodium dodecyl sulfate; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonate; EPR, electron paramagnetic resonance; ST-EPR, saturation-transfer EPR; V_2', second harmonic out-of-phase absorption ST-EPR signal; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography; EI, electron impact; FAB, fast atom bombardment; NMR, nuclear magnetic resonance; IR, infrared.

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