ON THE CONDUCTANCE HETEROGENEITY IN MEMBRANE CHANNELS FORMED BY GRAMICIDIN A

A Cooperative Study

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ABSTRACT The relative frequency of low-conductance variants of gramicidin A channels in lipid bilayers was determined in parallel experiments in two different laboratories. A common gramicidin stock solution was tested in both labs and, initially, gave rise to significantly different proportions (9% v. 23%) of "mini" channels in the two labs. The lipid and gramicidin solutions were exchanged to identify the source of the difference: When using solutions prepared in lab A (Andersen), lab B (Busath) observed 9% minis, consistent with the original findings in lab A; when using the gramicidin solution prepared in lab B, lab A observed 18% minis, consistent with the original findings in lab B. The experimental apparatus and analysis techniques are therefore not the source of the discrepancy; rather, the difference appears to stem from some factor(s) related to the gramicidin, lipid, and electrolyte solutions. It appears that the mini frequency cannot reflect intrinsic characteristics of the channel-forming peptide, but rather must, at least in part, reflect environmental modulations of channel properties. This has implications for the interpretation of multi-channel experiments on gramicidin A.

INTRODUCTION

It is generally accepted that the linear gramicidins, exemplified by gramicidin A, form channels whose conductances mostly fall within a narrow range. Reports also indicate that 25–50% of channels formed by purified gramicidin A can be variants ("minis") having conductances above or below the standard channel range (Busath and Szabo, 1981; also see figures in Hladky and Haydon, 1972; Bamberg et al., 1976; Prasad et al., 1982; Urry et al., 1984 *a,b*). This heterogeneity of channel conductances could be an intrinsic characteristic of the polypeptide channels due to stable conformational variants of the peptide. If this were the case, one would expect the relative frequency of mini occurrences to be fairly invariant among different laboratories. Mazet et al. (1984) and Russell et al. (1986) found, however, a significantly lower mini frequency with channels formed by valine gramicidin A (<10%). To test whether such a difference could be due to inadvertent contamination with other channel-formers, contaminants from the HPLC column, data analysis procedures, laboratory environment, the bilayer formation technique, the experimental chamber, the electrolyte solution, the lipid or lipid solvent, the gramicidin injection method, or the gramicidin solvent used, we undertook a cooperative study. Our results suggest that differences in observed mini channel frequency can arise from three of these factors: the gramicidin solvent, the saline solution, and the lipid sample. We conclude that the mini frequency depends on external factors and cannot be used to characterize the peptides per se.

MATERIALS AND METHODS

Gramicidins A, B, and C have previously been resolved by HPLC on columns of octadecyl-silica (Axelsen and Vogelsang, 1977; Urry et al., 1983), phenyl-silica (Koeppe and Weiss, 1981), or octyl-silica (Morrow et al., 1979). An example of the separation of natural gramicidin components on octyl-silica is shown in Fig. 1. The valine (A1, B1, and C1) and isoleucine (A2, B2, and C2) subspecies are clearly illustrated. The B2 peak has not previously been demonstrated in the literature.

For our studies, valine gramicidin A was doubly purified using an octyl-silica column at room temperature. 7 μl of methanolic (HPLC grade, Fisher Scientific Co., Pittsburgh, PA) gramicidin D (1 mg/ml; Sigma Chemical Co., St. Louis, MO) was injected into a 4.6 × 250-mm "Zorbax-C8" column (Dupont Co., Wilmington, DE). The column was eluted at 1.5 ml/min (pressure ~1,800 psi) using 80% methanol (HPLC grade; Fisher Scientific Co.) that had been filtered and degassed with helium. The gramicidins in the eluant were detected by UV absorbance at 280 nm. When the polarity of the carrier solvent is increased, the gramicidin A peaks are completely separated as shown in Fig. 2 *a*. The valine gramicidin A peak (between the hatch marks) was collected by hand, concentrated by the evaporation of solvent, and purified a second

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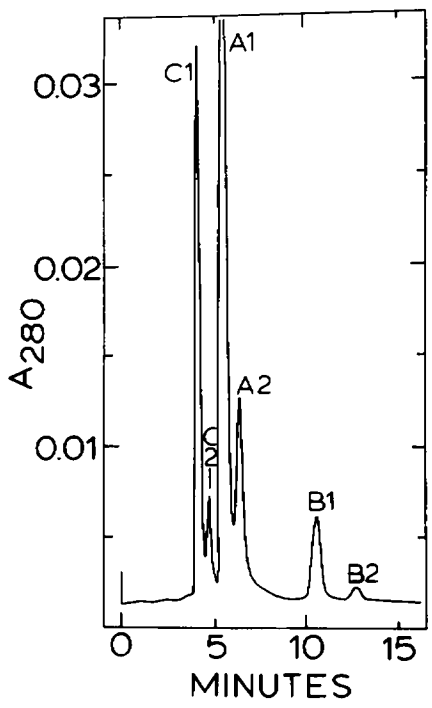


FIGURE 1 Illustration of the resolution of valine (A1, B1, and C1) and isoleucine (A2, B2, and C2) components of a gramicidin D mixture (83% methanol, 1.5 ml/min, 4.6 × 250-mm octyl-silica column, room temperature).

time on the same HPLC column using 81% methanol (Fig. 2 *b*). The final sample (~3 μg/ml in the column effluent), taken between the hatch marks in Fig. 2 *b*, was stored in glass with room air at or below room temperature for use in both lab A and lab B.

Lab A (Andersen)

Planar lipid bilayers were formed at 24°–38°C (usually 24°–27°C) by the method of Szabo et al. (1969) across a hole (area ≃1.6 mm^2) in a Teflon partition separating two Teflon chambers containing 5 ml of unbuffered 1.0 or 2.0 M NaCl (Suprapur grade from E. Merck through Manufacturing Chemists Co., Cincinnati, OH). The NaCl was roasted at 550°C for 24 h and stored over NaOH in evacuated desiccators, and the water was deionized milli-Q water (Millipore Corp., Bedford, MA). Except where noted, the aqueous solutions were made up on the day of the experiment. The membrane-forming solutions were 5% (wt/vol) glycerolmonoolein (GMO; Sigma Chemical Co.) in *n*-hexadecane (Wiley Organics, Columbus, OH) or 2% diphytanoylphosphatidylcholine (DPhPC; Avanti Polar Lipids, Inc., Birmingham, AL) in *n*-decane (Wiley Organics). DPhPC was purified by ion-exchange chromatography before use (e.g., Andersen, 1983). The doubly HPLC-purified valine gramicidin A was diluted 1:1,000 (for GMO) or 1:100 (for DPhPC) into ethanol (200 proof USP reagent quality from U.S. Industrial Chemicals Co., Tuscola, IL). A 5–20-μl aliquot of the diluted gramicidin was added to each aqueous phase after a bilayer was formed and the system was checked.

The single-channel measurements were done at 100 mV applied potential using the bilayer punch and on-line analysis algorithm described elsewhere (Andersen, 1983). The magnitude of the single-channel current transitions was estimated as the difference between the mean current levels for 20–50 ms records collected immediately before and after each open/close transition. No attempt was made to match channel-opening events with channel-closing events. The discrimination level to trigger the

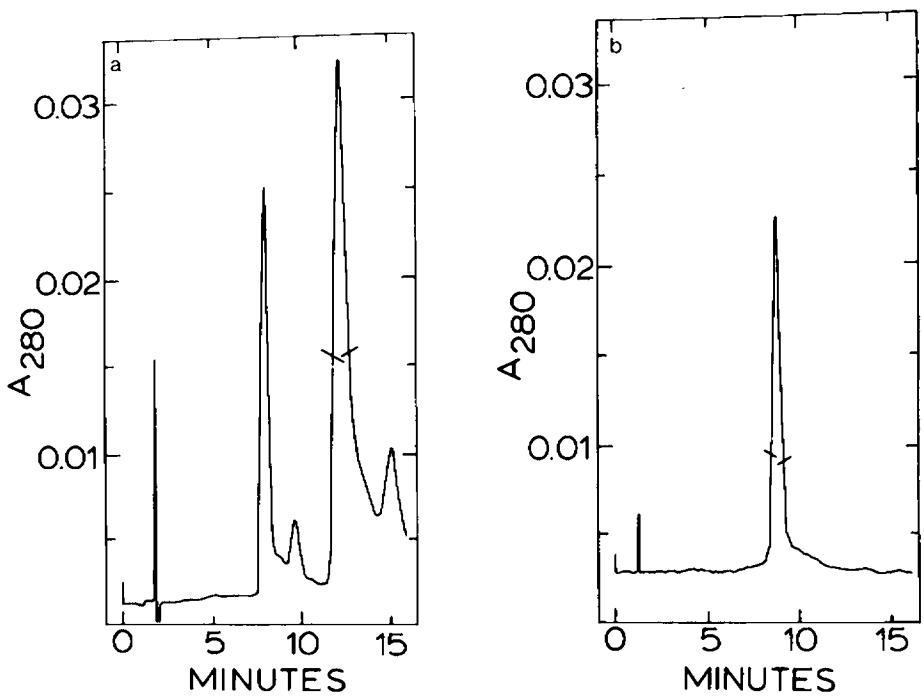


FIGURE 2 Purification of gramicidin A by HPLC on a 4.6 × 250-mm octyl-silica column eluted with methanol-water at 1.5 ml/min. (*a*) Purification and collection of Val^1-gramicidin A using 80% methanol elution. (*b*) Re-purification of the peak from *a* using 81% methanol elution. The resulting doubly purified Val^1-gramicidin A was collected as indicated, shipped in the elution solvent, and used for the single-channel experiments described in this article.

algorithm was set sufficiently low that the baseline current noise would trigger a significant number of "current transitions." This was done to ensure that low-amplitude current transitions would be incorporated into the sample and to provide an estimate of the instrumental rms noise. Each experiment consisted of 9–28 consecutive records of 400 current transitions. The histograms of current transitions for each record were analyzed and compared to the aggregate histogram, the histogram of current transitions for all records collected on a given day. Records were eliminated from the final data set if there was poor small-membrane stability, if there was an excess of current spikes, if the channel activity was too high, or if there was evidence for increased series resistance originating in the pipette. A total of eight records out of 70 were rejected from four GMO experiments for these reasons, whereas 11 out of 129 records were eliminated from 12 DPhPC experiments.

The determination of the number of standard and mini current transitions was done for each record, based on inspection of the amplitude histograms. The distinction between standard and mini current transitions was based upon two criteria. First, we sought a clear-cut delineation in the amplitude histogram between the major peak and the minis where there was zero, maybe one transition in the bin. Second, the width of the histogram section assigned to represent standard channels was compared with the width of the gaussian standard channel peak and assured to extend less than about three standard deviations in either direction from the mode of the peak. In the aggregate histograms, the width of the peak for the standard channels was assigned to be somewhat broader though, about eight standard deviations, because the large number of channels under consideration and the increased spread in the peak due to varying environmental factors resulted in a significant number of standard transitions in the fourth deviation from the mean. For each record the number of transitions falling within the effective limits of a normal distribution, t_s, and the number falling outside of these limits, t_m, were counted.

Lab B (Busath)

Lipid bilayers were formed using a solution of 5% (wt/vol) GMO (Nu Chek Prep, Inc., Elysian, MN) in hexadecane (Gold Label, Aldrich Chemical Co.). 1.0 M KCl (J. T. Baker Chemical Co., Phillipsburg, NJ), baked at 600°C for 4–6 h, was prepared using water purified using a Millipore Corp. filtration unit. The solution was then filtered (0.2-μm pores) using a Nalgene disposable filter. For all experiments except those listed in Table V, an aliquot of the purified gramicidin A sample was diluted 1:1,000 into ethanol (200 proof, Rossville Gold Shield, USP quality; IMC Chemical Group, Inc., Terre Haute, IN; or Aaper Alcohol and Chemical Co., Reagent Quality, Louisville, KY). For the experiments in Table V, gramicidin purified in lab B using HPLC was diluted and stored in methanol (HPLC grade; J. T. Baker Chemical Co.). Acetaldehyde, formaldehyde, and aminoethanol were commercially available reagent grade products used without purification.

Bilayers were painted on the 40-μm aperture of a polyethylene pipette inserted into a Teflon chamber using the method of Szabo et al. (1969). 2-μl aliquots of the diluted gramicidin A solution (1.5 ng/ml) were then introduced into the Teflon chamber which contained ~3 ml saline. The membrane current was measured for 20-min periods after membrane formation. Channels formed at a rate of ~0.1–0.3/s, yielding 240–720 transition measurements per record. Some membranes were rejected routinely due to unipolar non-channel noise, but the records were not selected on the basis of the channel appearance (e.g., the apparent frequency of minis). Data were analyzed for current transitions using a double-threshold algorithm which recognizes transitions when the difference between adjacent current measurements exceeds an upper threshold and defines the ends of flat segments before and after transitions as points that differ from their neighbors by less than a lower threshold. The amplitudes of all the points between the start and end of a flat segment are averaged to get the segment amplitude. The channel current transitions are defined as the differences between adjacent segments. The lower threshold is set at the peak-to-peak noise level. By digitizing at a rate equal to three times the low-pass filter cutoff frequency (typically 100 Hz, the start- and endpoints of segments were clearly defined by this approach. All computer determinations were examined carefully for missed transitions and were corrected manually when deemed appropriate. For each record, positive transitions were combined with the reverse of negative transitions to produce single channel current histograms. Each histogram was evaluated by fitting a normal curve by eye to the standard channel peak. For each histogram, the number of transitions falling within two standard deviations of the normal curve mean, t_s, and the number falling outside (above and below) the normal peak, t_m, were counted.

The percentage of minis is used throughout this paper as an index of heterogeneity. It will be referred to as the mini frequency and is calculated for each record as

$$f_m = 100\, t_m/(t_s + t_m)\,.$$

The counting error or standard error of f_m, SE(f_m), was estimated by the propagation of errors method assuming that the mini and standard channel occurrence counts, c_m and c_s, were half the mini and standard transition counts and were results of independent stationary Poisson processes:

$$\mathrm{SE}(f_m) = 100\, c_m\, c_s\, \mathrm{sqrt}(1/c_s + 1/c_m)/(c_s + c_m)^2\,.$$

RESULTS

Lab A (Andersen)

Single-channel current traces obtained in lab A using materials from lab A are shown in Fig. 3. Examination of the 26 channels in the record shows that only two are minis. This results is typical for measurements made in lab A. A histogram of channel current transitions from a similar experiment is demonstrated in Fig. 4. It includes data from 13 small membranes obtained on the same day. It is very difficult to judge from the histogram alone what the mini frequency is, but the relative dearth of minis is quite obvious. In this case the mini frequency was 5.5%.

The day-to-day behavior of the mini frequency is demonstrated in Table I which describes data obtained in lab A. Each line of the table summarizes experiments done on a single day using several different large membranes. Both GMO and DPhPC membranes were used. From each large membrane, a number of small membranes (column 1) were punched out (Andersen, 1983). A single record containing ~200 transitions was collected for each small membrane. The data in Table I demonstrate two points. First, lab A is consistently able to obtain a much lower mini frequency (<10%) than has been reported from most other laboratories. Second, even though the mini frequency was low in lab A, it was variable. It did not vary substantially from one large membrane to the next on a given day but it did vary by a factor of three from day to day. This day-to-day variation in mini frequency, f_m, is not due to counting error, SE(f_m), which is <2% for these experiments. For any one membrane, the variations in mini frequency are random, as opposed to time-dependent. The variation is therefore not due to a degradation process. Studies in lab A indicate, however, that if the same

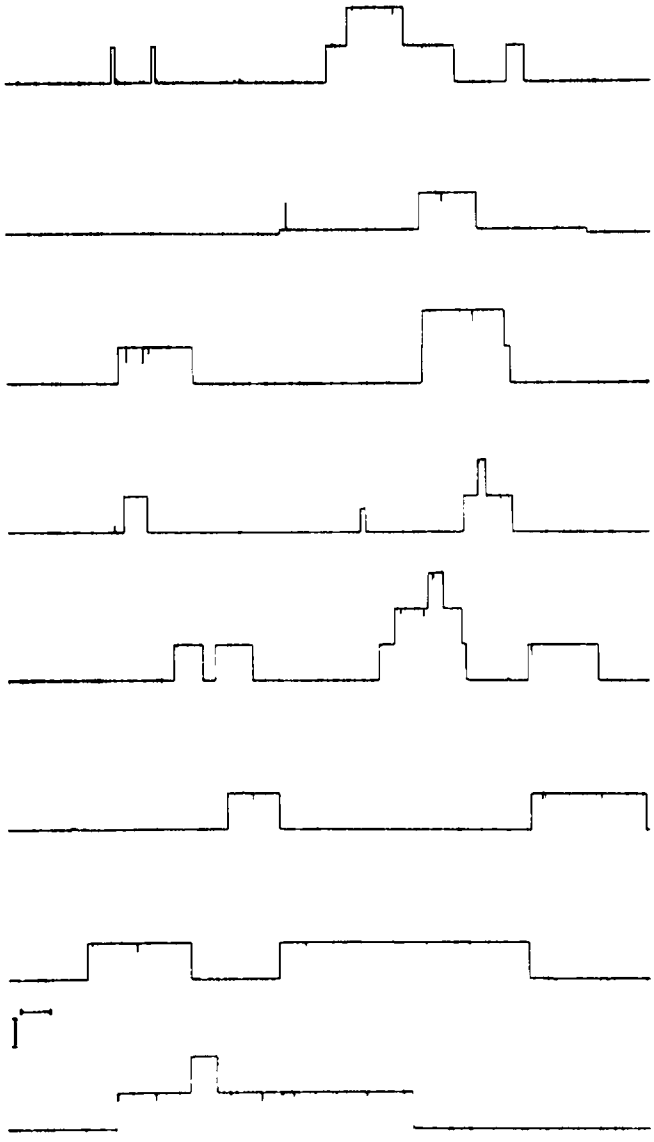


FIGURE 3 Illustration of single-channel activity obtained in Lab A using the gramicidin dilution prepared in Lab A. The record depicts eight segments, each lasting 22.5 s, of data obtained using a single patch of GMO membrane. Intervening segments with no or very little channel activity have been deleted. Note the uniform amplitude of most of the channels. The second and fourth segments contain typical mini channels. The mini frequency for the whole data set was 8.2%. The calibration bars denote 2.0 pA vertically and 1.0 s horizontally. 1.0 M NaCl, filter cutoff 100 Hz.

electrolyte solution was used on successive days, the mini frequency increased on the later days (Table II). The changes in the shape of the current amplitude histograms with aging of the electrolyte are illustrated in Fig. 5, *a* and *b*. Evidently, the mini frequency is modulated by factors in the environment, and the elecrtrolyte solution is one source of this variable.

The mini frequency could also be increased by elevation of the temperature. Fig. 5 *c* shows the channel conductance histogram for experiments at 37°C–38°C, where the mini frequency was 20–24%. The general appearance of the

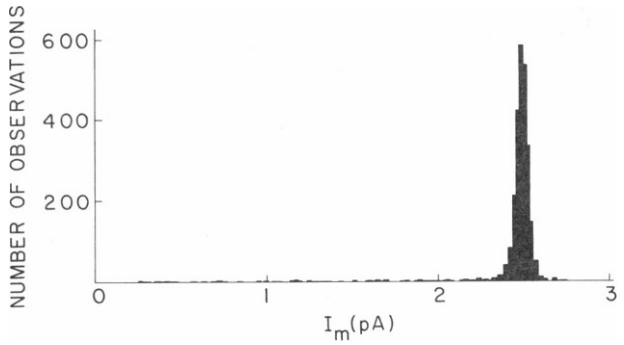


FIGURE 4 Amplitude histogram based upon 2622 current transitions obtained in 13 consecutive records in Lab A using Lab A materials. There were 2,472 standard channel transitions (2.295 to 2.637 pA), and 150 "mini" channel transitions: 131 in the range 0.269–2.271 pA and 19 in the range 2.661–3.271 pA. The mini frequency for the aggregate histogram was 5.7%. For the individual records, this number ranged from 1.8 to 12.2%. The average current for the standard channels was 2.471 ± 0.044 pA (mean ± SD). The average current for the standard channels in the individual records ranged from 2.439 to 2.491 pA and the standard deviations ranged from 0.035 to 0.064 pA. The instrumental contribution to the standard deviation was estimated to be ≈0.05 pA, based on 122 baseline fluctuations detected as transitions between 0 and 0.195 pA. GMO, 1.0 M NaCl.

mini channel range is similar in the three, histograms of Fig. 5. The increased mini frequency in Fig. 5, *b* and *c* results from a larger relative frequency of the mini events, not from the emergence of new peaks.[1]

Lab B (Busath)

The results obtained in lab B using materials from lab B yielded a higher mini frequency than obtained in lab A, 22.6% (line 1 of Table III). This result was consistent with all experiments (between 50 and 100) done previously in lab B with other samples of gramicidin A. The gramicidin and lipid solutions used in lab A for the experiments of Table I were therefore flown to lab B and vice versa for independent testing. The mini frequency obtained in lab B, using combinations of materials from both labs is reported in Table III (lines 2–4). The greatest mini frequency was found when using the gramicidin or lipid solutions prepared in lab B (21.2% and 33.4%). When using the lipid and gramicidin solutions from lab A, however, the mini frequency was found to be only 5.2% (line 4), a value distinctly lower than previous experience in lab B and consistent with values typically found in lab A. This was reproduced using lipid only from lab A (lines 5–7). Again,

[1]The increased width of the main peak in Fig. 5, *b* and *c* (compared with the main peak in Fig. 5 *a*) was observed in the measurements from the small bilayers making up the aggregate histogram and was not, therefore, a consequence of a drift in the standard channel conductance. From these experiments, it appears that conditions that promote the formation of mini channels also leads to a larger than normal heterogeneity in the major peak. On the other hand, the experiments in lab B (Table IV) yielded variable mini frequencies but consistently narrow main peak widths.

TABLE I
MINI FREQUENCY DATA OBTAINED IN LAB A USING LAB A MATERIALS

	No.	t_m	t_s	f_m	SE(f_m)
				%	%
I	13	141	2,481	5.4	0.6
	23	273	3,554	7.1	0.6
	9	266	1,314	16.8	1.3
	17	319	2,237	12.5	0.9
II	10	236	1,609	12.8	1.1
	8	161	1,882	7.9	0.8
	27	598	4,431	11.9	0.6
	19	805	6,010	11.8	0.6
III	10	258	4,574	5.3	0.5

Each line represents work from 1 d using electrolyte solutions made that day and includes data from several membranes. The number of patches summarized in each line is given in the first column (No.). The second column (t_m) contains the total number of mini transitions observed; the third column (t_s), the total number of standard transitions observed. The fourth column contains the mini frequency, f_m. The fifth column gives the standard error of the mini frequency. (I) 5% GMO in hexadecane, 1.0 M NaCl. Standard channels were those with conductances that varied < ~1.5 pS from the mean values. The mean standard channel conductances ranged from 24.4 to 26.7 pS for the 62 experiments. Standard deviations for the main peak in the histograms varied between 0.3 and 0.6 pS. (II): 5% DPhPC in decane. Standard channels were those with conductances that varied < ~1.0 pS from the mean values. The mean standard channel conductance ranged from 12.5 to 15.8 pS for the 29 experiments in 1.0 M NaCl (first and fourth lines), and 14.4 to 17.4 pS for 35 experiments in 2.0 M NaCl (second and third lines). Standard deviations for the main peak in the histograms varied between 0.2 and 0.6 pS. (III) 5% DPhPC in decane, 1.0 M NaCl. Mini frequency obtained in lab A using undiluted gramicidin A. About 100 nl of stock gramicidin was injected into the chamber. Standard channels were those with conductance that varied, i.e., were ~1.05 from the mean values. Mean standard channel conductances varied between 12.5 and 13.6 pS. Standard deviations for the main peak in the histograms varied between 0.2 and 0.3 pS. V_m = 100 mV; temperature = 24°–27°C.

the mini frequency obtained using the gramicidin solution from lab A was lower (9.4% and 11.3%) than that produced by the solution from lab B (19.3%). If the experiments with gramicidin from lab B (1, 3, and 5) are compared using the unpaired t test to those with gramicidin and lipid from lab A (4, 6, and 7), the former are found to yield a significantly higher mini frequency (25.1% vs. 8.6%; SD = 7.4% and 3.1%, respectively; $P<0.02$). Conversely, when the gramicidin dilution from lab B was examined in lab A (line 8), the mini frequency was 18.4%, which is higher than typically observed in lab A (compare Table I). The channel current histograms from experiments three and four of Table III are compared in Fig. 6. The greater frequency of minis using the gramicidin prepared in lab B is evident in the upper histogram. These experiments were performed in the order listed, all using the same electrolyte. They indicate that not only the electrolyte but also the gramicidin solution can contribute to the variable factor(s). The only difference we can identify between the two samples of gramicidin used here

TABLE II
MINI FREQUENCY IN LAB A AS A FUNCTION OF THE AGE OF THE ELECTROLYTE SOLUTION

Day	No.	t_m	t_s	f_m	SE(f_m)
				%	%
1	19	805	6,010	11.8	0.6
2	12	934	2,258	29.3	1.1
4	15	1,007	2,971	25.3	1.0
8	14	893	2,912	23.5	1.0

1.0 M NaCl was made on day 1, and stored in a glass-stoppered Pyrex bottle at room temperature (20°–24°C). Experiments were done at 25°C (±1%) with the same membrane-forming solution and gramicidin dilution on the day indicated in the first column. The other columns are the same as Table I. Standard channels were those with conductances that varied < ~1.0 pS from the mean value. Mean standard channel conductances varied between 12.42 and 13.49 pS. Standard deviations varied between 0.15 and 0.35 pS for the experiments on day 1, and 0.28 and 0.87 pS for the experiments on days 2–8. 1.0 M NaCl, 100 mV.

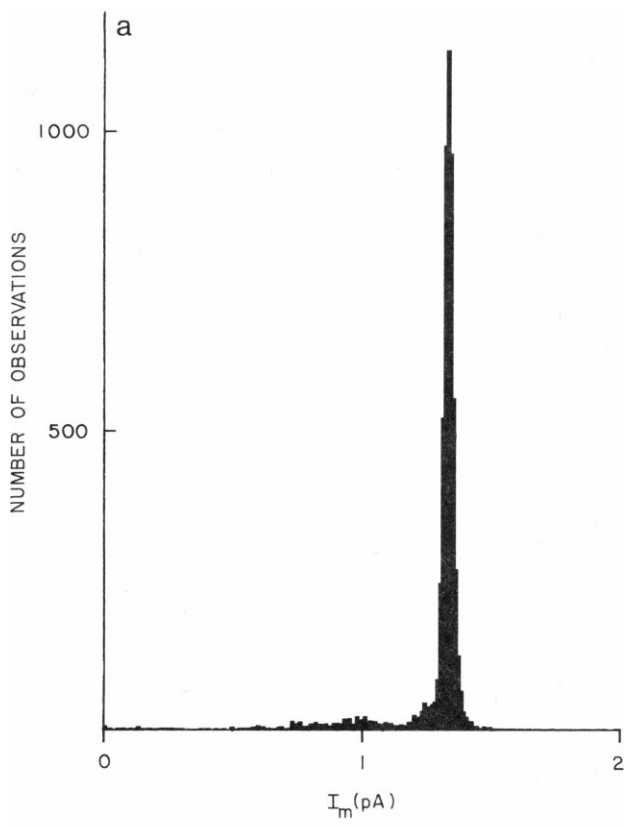
was the ethanol used to dilute them. Furthermore, in lab B the mini frequency did not vary as a function of the age of the electrolyte composition. The mini frequency varied between 32 and 39% in experiments parallelling those summarized in Table II (data not shown). The difference between the two laboratories cannot be ascribed solely to progressive contamination of the electrolyte solutions.

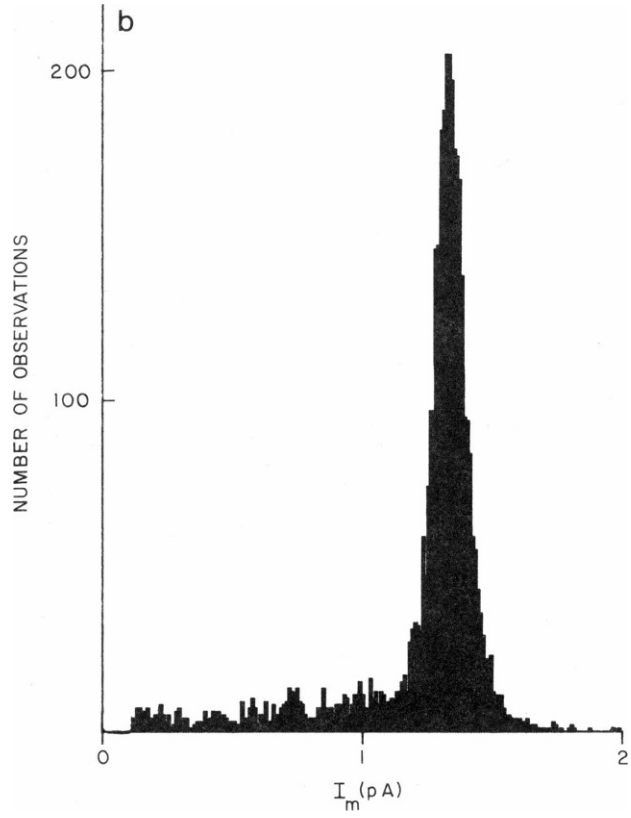
Further tests of the hypothesis that ethanol contains the contaminant also yielded inconclusive results. We mea-

TABLE III
MINI FREQUENCY DATA FROM EXPERIMENTS PERFORMED IN LAB B (LINES 1–7) AND LAB A (LINE 8)

	Gramicidin dilution	Lipid source	t_m	t_s	f_m	SE(f_m)
					%	%
1	Lab B	Lab B	58	199	22.6	3.7
2	Lab A	Lab B	75	279	21.2	3.1
3	Lab B	Lab A	222	442	33.4	2.6
4	Lab A	Lab A	66	1,202	5.2	0.9
5	Lab B	Lab A	148	617	19.3	2.0
6	Lab A	Lab A	37	290	11.3	2.5
7	Lab A	Lab A	13	125	9.4	3.5
8	Lab B	Lab A	251	1,116	18.4	1.5

Columns 2 and 3 indicate the lab where the gramicidin and lipid samples respectively were prepared. Columns four through seven are the same as in Table I. Lines 1–7, each line contains data from a single experiment; 5% GMO in hexadecane. In each case (except line 6) standard channels were found to be best defined as those having conductances within the approximate range 44.2 to 46.6 pS. The mean standard channel conductance ranged from 44.7 to 45.9 pS. The main peak standard deviation varied between 0.19 and 0.37 pS except for line 6 (0.80 pS); V_m = 100 mV; temperature = 23°C; 1.0 M KCl. Line 8, data from 6 small membranes in two large membranes are totaled together. These experiments were done 20 mo after those from lines 1–7, the gramicidin dilution from lab B having been stored at 0°C in the interim. Standard channel conductance varied between 13.2 and 15.4 pS. Standard deviations for the main peak in the histograms varied between 0.4 and 1.0 pS. DPhPC; V_m = 100 mV; 24°–27°C; 1.0 M NaCl.





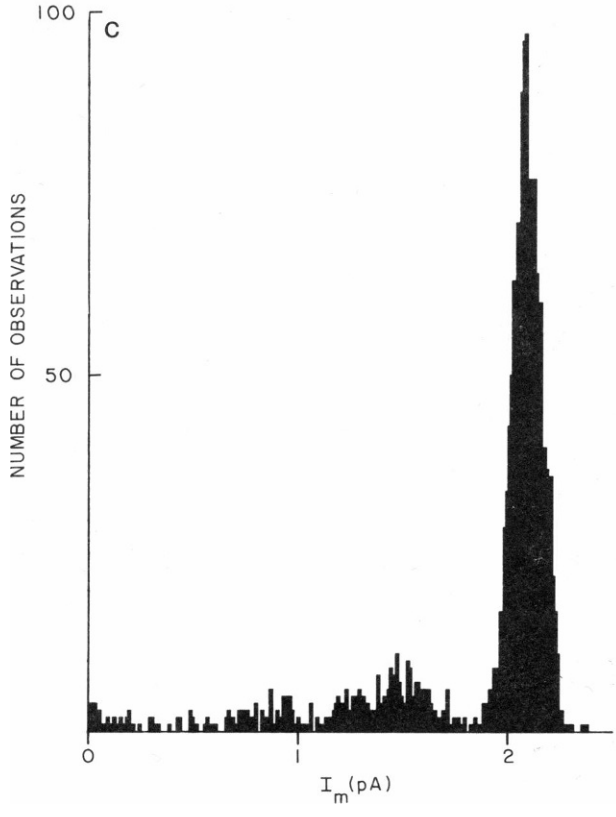


FIGURE 5. Amplitude histograms obtained in lab A using lab A materials. DPhPC, 1.0 M NaCl. (*a*) Results with freshly made electrolyte at 25°C. The histogram contains 5834 current transitions collected in 19 individual measurements, the current amplitudes were determined from 40 ms segments just before and after the transitions, the filter frequency was 50 Hz. There were 5,265 standard channels transitions (between 1.212 and 1.440 pA), and 569 "mini" channel transitions: 548 in the range 0.101–1.202 pA, and 23 in the range 1.450–2.0 pA. The mini frequency for the aggregate histogram was ~9.8%. For the individual records, this number varied between 6.9 and 19.2%. The average current for the standard channel transitions was 1.326 ± 0.032 pA. The average current for the standard channel transitions varied between 1.316 and 1.349 pA, and the standard deviations varied between 0.015 and 0.035 pA. The instrumental contribution to the standard deviation was 0.03 pA. (*b*) Results with an 8-d-old electrolyte at 25°C. The histogram contains 3802 current transitions collected in 14 individual measurements, other conditions as in *a*. There were 3,031 standard channels transitions (between 1.118 and 1.530 pA), and 771 "mini" channel transitions: 620 in the range 0.101–1.107 pA, and 151 in the range 1.550–2.0 pA. The mini frequency for the aggregate histogram was ~20.3%. For the individual records, this number varied between 14.1 and 35.5%. The average current for the standard channel transitions was 1.331 ± 0.074 pA. The average current for the standard channel transitions varied between 1.308 and 1.364 pA, and the standard deviations varied between 0.042 and 0.080 pA. The instrumental contribution to the standard deviation was ≃0.05 pA. (*c*) Results with a fresh electrolyte at 37.5°C. The histogram contains 1,883 current transitions collected in 10 individual measurements, other conditions as in *a*. There were 1,443 standard channel transitions (between 1.953 and 2.255 pA), and 440 "mini" channel transitions: 435 in the range 0.101–1.943 pA and 5 in the range 2.265–2.5 pA. The mini frequency for the aggregate histogram was ~23.3%. For the individual records, this number varied between 16.9 and 34.4%. The average current for the standard channel transitions was 2.093 ± 0.068 pA. The average current for the standard channel transitions varied between 1.949 and 2.158 pA, and the standard deviations varied between 0.045 and 0.150 pA. The instrumental contribution to the standard deviation was ~0.05 pA.

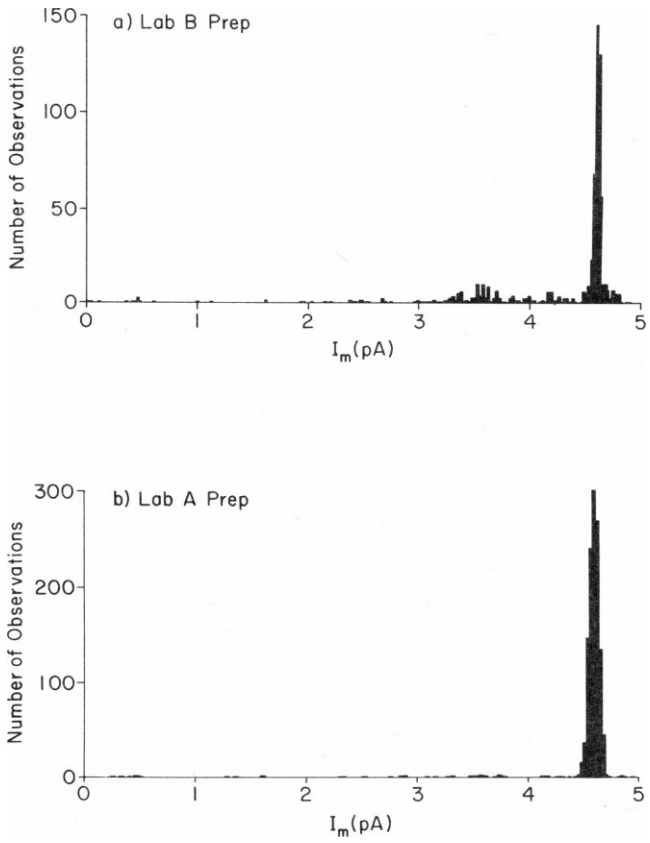


FIGURE 6. Single channel current histograms obtained in lab B using GMO from lab A and gramicidin A either from lab B (*a*) or from lab A (*b*). The absolute values of both negative and positive transitions from a single bilayer have been binned together in each case. (*a*) Data from the experiment of Table II, line 3. 664 transitions of which 442 were standard channels (4.418–4.662 pA) having 4.594 pA mean current (SD = 0.034 pA). Of the remaining 222 minis (33.4%), 202 conducted below 4.418 pA (one should have been excluded because it was below the resolution limits) and 20 conducted between 4.662 and 4.916 pA. 1.0 M KCl. (*b*) Data from Table II, line 4. 1,268 transitions, 1,202 of which were standard channels (4.418 pA $< i <$ 4.662 pA) having mean current of 4.589 pA (SD = .037 pA). Of the 66 "minis" (5.2%), six had currents higher than standard (4.662 pA $< i <$ 4.980 pA), 58 had currents lower than standard, and two should have been excluded because they were below the limits of resolution. 1.0 M KCl, filter cutoff 100 Hz.

TABLE IV
MINI FREQUENCY FOR VARIOUS PREPARATIONS OF THE GRAMICIDIN A DILUTION

Sample prep. No.	Ethanol brand	Sample age	t_m	t_s	f_m	SE(f_m)
		d			%	%
1	RGS	1	148	617	19.3	2.0
		1+	40	66	37.7	6.7
2	RGS	1	18	148	10.8	3.4
		1+	28	193	12.7	3.2
		1++	109	334	24.6	2.9
		2	313	436	41.8	2.5
		2+	146	230	38.8	3.6
3	AAP	1	14	141	9.0	3.3
		3	132	429	23.5	2.5
4	AAP	1	47	233	16.8	3.2
		2	153	91	62.7	4.4
		2+	70	41	63.1	6.5

Experiments performed in lab B using lipid (GMO) from lab A. Column 1 gives the number of the 1,000-fold dilution preparation of gramicidin A. Column 2 indicates the brand of ethanol used to dilute the gramicidin. RGS, Rossville Gold Shield; AAP, Aaper brand. Column 3 indicates when the experiment was performed. The dilution was prepared on day 1. The gramicidin stock being diluted had been prepared several weeks previously. All the experiments in this table were performed within a 3-d period. The other columns are the same as in Table I. The mean standard channel conductances varied between 44.29 and 46.48 pS. The main peak standard deviations varied between 0.26 and 0.53 pS. 1.0 M KCl, 100 mV.

sured the mini frequencies obtained by direct application of nanoliter quantities of the purified gramicidin stock solution to the experimental chamber without prior dilution into ethanol. When done in lab B a large mini frequency was obtained (f_m = 43.2%; SE(f_m) = 4.2%; t = 122; t_s = 160). However, the same experiment performed later in lab A, again with the same stock sample, yielded a mini frequency of only 5.3% (last line of Table I). The experiment in lab B could suggest a contaminant in the HPLC eluant, but the subsequent finding in lab A rules it out. Two different sources of ethanol (freshly opened) were compared in lab B. The results are shown in Table IV. The mini frequency varied considerably for all four gramicidin preparations. The Aaper brand of ethanol (a fresh bottle) showed no consistent improvement over our standard brand (Rossville). There is, nevertheless, a clear trend: the mini frequency increased about twofold between the first experiment with a fresh dilution and the subsequent experiments for each of the four preparations.

If typical ethanol contaminants were responsible for mini channels, addition of a large concentration of contaminant to the gramicidin solution should enhance the mini frequency. In the purification of ethanol, traces of benzene sometimes remain. After purification, ethanol slowly oxidizes and small amounts of acetaldehyde can accumulate. Also, ethanol is hygroscopic and normally contains 2–3% water. In one experiment in lab B, we diluted gramicidin with 90% ethanol, 10% benzene. After waiting ~1 h, the mini frequency was determined and found to be 17.5% ($t_m + t_s$ = 627; SE(f_m) = 2.1%), similar to or less than the usual level in lab B. To determine whether aldehydes could cause minis, we measured the mini frequency after adding 1% acetaldehyde, 1% formaldehyde, 12% formaldehyde, or 1% aminoethanol to the gramicidin sample. Aminoethanol was expected to absorb free aldehydes. The results are given in Table V. There is no consistent increase in mini frequency with incubation in aldehydes or decrease with incubation in aminoethanol from the usual values observed in lab B. The control samples yielded lower mini frequencies than are typical for lab B. Subsequent experiments with the same conditions as in the control continue to yield mini frequencies of 25–50%. In two experiments, the effect of incubating gramicidin A with 10% water (90% ethanol)

TABLE V
MINI FREQUENCIES FROM GRAMICIDIN A INCUBATED STARTING ON DAY 1 WITH ACETALDEHYDE, FORMALDEHYDE, OR AMINO ETHANOL

	Days 2–11			Days 21–28		
	$t_m + t_s$	f_m	SE(f_m)	$t_m + t_s$	f_m	SE(f_m)
		%	%		%	%
1% Acetaldehyde	188	50.5	5.2	149	34.2	5.5
1% Formaldehyde	918	15.7	1.7	276	23.2	3.6
12% Formaldehyde	130	26.2	5.5	404	15.1	2.5
1% Aminoethanol	179	32.4	4.9	55	32.7	8.9
Control	662	19.8	2.2	217	12.0	3.1

In the control sample, the gramicidin was dissolved in 100% methanol. 1.0 M KCl, 100 mV.

was measured in lab B. On day 1, the mini frequency was measured as 11.0 ± 3.3% and on day 3, 11.5 ± 1.3% using a sample diluted in Aaper ethanol. This result is not substantially different from those reported for water-free sample in Table IV. Thus none of the obvious ethanol contaminants could be demonstrated to affect the mini frequency.

The lipid solution also seems to be responsible for some of the variability in the mini frequency. Comparison of lines 2 and 4 of Table III shows that a reduced mini frequency was obtained in lab B by merely switching to lipid from lab A. However the finding that the gramicidin sample prepared in lab B with the lipid from lab A yields a high mini frequency in either lab (lines 3, 5, and 8 of Table III) argues that the gramicidin solution also contains a variable factor. Thus there appear to be multiple sources of mini-inducing factors.

DISCUSSION

The heterogeneity of single-channel conductances in amplitude histograms is due to (*a*) the occurrence of well-resolved differing conductance states, i.e., mini channels; and (*b*) dispersion in the main peak. The main peak may be composed of unresolved substates and can be broadened by uncontrolled experiment-to-experiment and intra-experiment variations in the average single-channel conductance for the standard channels. In this paper we focus primarily on the mini channels.

The determination of the mini frequency is subjective, because there is no absolute transition between the major peak and regions of the histograms which obviously represent minis and because minis may "hide" in the standard channel peak. However, in practice, it seems to be quite feasible to divide channels into two categories, provided that the standard peak is narrow and distinct, as it usually is in our hands.

Our principal conclusions are that mini channels are formed by valine gramicidin A and that the mini frequency depends on the solutions used for the experiment as well as other experimental factors (temperature and the age of the electrolyte solution). We do not understand the basis for the variability in the mini frequency, but we believe that the observation of differences between our labs for a single purified sample of gramicidin A demonstrates that the mini frequency cannot be an intrinsic characteristic of the gramicidin molecule itself; it somehow also reflects the experimental conditions.

The existence and mechanism of formation of valine gramicidin A mini channels are of interest for several reasons, irrespective of the molecular mechanism(s) underlying the observed phenomena. First, a conducting channel is an easily monitored conformational state/assembly of the gramicidin A molecule. Studies of mini channels should thus be relevant for understanding the folding and insertion of polypeptides into lipid bilayers. Second, mini and standard channels are formed by the same molecular species. Studies of minis could thus elucidate the control mechanisms underlying ion movement through transmembrane channels. Third, at least one of the rate constants for ion movement through the channel, and probably the ion affinity (Busath and Szabo, 1984) must be different for mini channels compared with standard channels. The interpretation of data obtained in multi-channel studies will thus be uncertain because the results of such studies reflect the ensemble averaged behavior. Examples of studies that could be affected by this factor include reversal potential measurements (e.g., Myers and Haydon, 1972; Eisenman et al., 1976), many-channel current–voltage characteristics (e.g., Eisenman et al., 1980), tracer flux measurements in planar lipid bilayers (e.g., Schagina et al., 1978, 1983; Procopio and Andersen, 1979), equilibrium ion binding to channels in lipid vesicles (Veatch and Durkin, 1980), measurements of water permeability of the channel using streaming potentials or voltage-induced osmosis (Rosenberg and Finkelstein, 1978; Levitt et al., 1978), spectroscopic measurements on gramicidin channels in micelles (e.g., Urry et al., 1980; Hinton et al., 1982) or lipid vesicles (Monoi, 1985), and measurements of dielectric relaxation using gramicidin channels in lipid structures (Henze et al., 1982). One could arrive at erroneous conclusions regarding the permeability characteristics of the (standard) gramicidin A channels if there were a large fraction of mini channels in the channel population. It may, for example, be difficult to determine whether an observed multi-component behavior reflects multiple ion occupancy or the existence of several channel populations.

Many of the characteristics of mini channels have been described by Busath and Szabo (1981, 1984). The mini frequency was relatively invariant with respect to gramicidin concentration, composition of the membrane-forming solution, aqueous electrolyte composition, and membrane area. It was further found that there were spontaneous transitions between standard and mini channels, and that the average single-channel lifetimes for minis and stan-

dards were comparable. It was concluded that the mini channels represent comparatively minor variations of the dimeric $\beta^{6.3}$-helical structure proposed by Urry (1971). The present results provide additional support for this view. The observation of mini channels in both laboratories provides very strong evidence that most, if not all, mini channels are formed by valine gramicidin A. Possible molecular mechanisms underlying the variations in channel conductances, which have been suggested previously, include: minis could have variant orientations of the amino acid side chains; the backbone structure could have minor misalignments held in place by neighboring hydrogen bonds; and a foreign molecule could bind to a side chain or to one end of the channel and influence the electric field or the channel conformation (Busath and Szabo, 1981).

Between our labs, the differences in mini frequencies have been too large to be the consequence of statistical fluctuations due to Poisson counting errors. Other possible differences in routine handling by the two labs are ruled out by the direct comparison of the two specimens in both labs A and B. The present results suggest that something in the environment of the gramicidin molecules may have a long lasting effect on the channel conductance. For simplicity, we will refer to this as a "contaminant." This contaminant could be present in the gramicidin sample, the lipid solution, or the electrolyte solution. In principle, the mechanism of action of the contaminant could be direct through binding to the channel molecules or indirect through an effect on their conformation. We also note that the day-to-day variation in the mini frequency in both labs A and B is greater than can be accounted for by the counting error and we were unable to consistently assign either the lipid or the gramicidin solution as the sole contaminant source. This raises doubts about a simple contaminant hypothesis and suggests that other factors may also be involved. It is of interest in this respect that results in lab A indicate additional factors influencing the mini frequency: aging of the electrolyte and increased temperature. The latter result confirms that of Urry et al. (1984 *a*).

Urry et al. (1984 *b*) predicted and observed lower mini frequencies with Leu^5-gramicidin A (versus the standard Ala^5-gramicidin A). The prediction was based on the notion that mini channels could differ from standard channels in the rotational positions of some of their side chains, particularly the bulky tryptophan No. 11 near the channel entrances. The Leu at position 5, being larger than the Ala found in native gramicidin, should reduce the number of rotameric conformations accessible to Trp^{11} from four to two (Venkatachalam et al., 1984). A twofold reduction of the number of conformations accessible to the monomer (estimated for normal gramicidin as 384 by Venkatachalam and Urry, 1983), could have a substantial effect on the overall mini frequency. However, the results of our study raise the possibility that the decreased mini frequency with Leu^5-gramicidin A could have resulted from inadvertent differences in the handling of the samples.

Conformational energy calculations suggest that some of the different rotameric states could be sufficiently long-lived to account for the stability of the conductances of individual channels (Urry et al., 1981; Venkatachalam and Urry, 1983). Indeed, though some interconversions between standard and mini channels have been observed, they are so infrequent as to argue that the proposed rotameric states must be separated by very large energy barriers, ~90 kJ/mol (Busath and Szabo, 1981). Barriers of this magnitude have been calculated for some of the interconversions between rotameric conformations (Urry et al., 1981). The rarity of the interconversions would also be consistent with other mechanisms, such as backbone misalignments stabilized by hydrogen bonds.

We have not been able to identify the molecular basis for the mini channels. Experiments with some expected ethanol impurities such as benzene, formaldehyde, and acetaldehyde and water failed to demonstrate any modulation of the mini frequency. Experiments in lab A indicate that the electrolyte age affects the mini frequency but this could not be confirmed in lab B. Some experiments in lab B indicated that the lipid solution could contain impurities, but others contradicted this hypothesis. We likewise cannot determine whether the residual mini percentage in lab A ($\simeq$8%) is an inherent characteristic of the polypeptide dimer or whether it reflects the persistent effects of putative impurities. We feel, however, that our results have established that linear gramicidins are very sensitive to the sample handling procedures. There is a reproducible difference between the mini frequencies observed in our labs. The mini frequency is therefore an elusive parameter, with unknown relation to the characteristics of the peptide forming the channels. Until the basis for the differences we observe can be ascertained, measurements of changes in the mini frequency should be interpreted cautiously because they may not reflect changes in the intrinsic properties of the channel structure.

We wish to thank Dr. G. Szabo for the use of facilities at "lab B" and for helpful discussions and encouragement. O.S. Andersen wishes to thank P. Landa, R. Levene, E. Narcessian, and L. Peart for technical assistance. D. D. Busath wishes to thank Dr. William Suggs for helpful discussions and G. Hemsley for technical assistance.

This work was supported by National Institutes of Health grants GM-33361 (D. D. Busath), GM-34968 (R.E. Koeppe II), GM-23142 (O. S. Andersen), and HL-24820 (G. Szabo).

Received for publication 28 March 1986 and in final form 7 August 1986.

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