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Gramicidin K, a New Linear Channel-Forming Gramicidin from *Bacillus brevis*†

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ABSTRACT: A new gramicidin has been isolated from a commercial mixture of gramicidins A, B, and C. This new molecule, designated gramicidin K, contains formyl and ethanolamine blocking groups, has a molecular weight ~20% higher than gramicidin A, and is strongly retained on reversed-phase liquid chromatographic columns. Gramicidin K can be resolved into two components, one of which contains tyrosine. In lipid bilayer membranes, both components form channels of considerably longer lifetime and somewhat lower conductance than gramicidin A. Gramicidin K appears to be a lipopeptide that consists of a fatty acyl chain attached to the ethanolamine of gramicidin A.

The Dubos strain of *Bacillus brevis* (ATCC 8185) synthesizes the polypeptide antibiotics tyrocidine and linear gramicidin (Hotchkiss, 1944) by a nonribosomal polyenzyme template mechanism (Lipmann, 1973, 1980). The polyenzyme systems produce peptides of defined length, but the sequence fidelity of the process is somewhat lower than for ribosomal protein synthesis, and so some heterogeneity of the products is observed. The linear gramicidins, for example, are heterogeneous at positions 1 (valine or isoleucine) and 11 (tryptophan, phenylalanine, or tyrosine) in the sequence of 15 amino acids (Gregory & Craig, 1948; Ramachandran, 1963; Sarges & Witkop, 1965a–c). This heterogeneity is presumably due to a relaxed enzymatic specificity for the amino acid to be incorporated at each of these positions in the peptide (Lipmann, 1973). The major linear gramicidin produced by *B. brevis* is valine-gramicidin A, which has the amino acid sequence formyl-L-Val_1-Gly_2-L-Ala_3-D-Leu_4-L-Ala_5-D-Val_6-L-Val_7-D-Val_8-L-Trp_9-D-Leu_{10}-L-Trp_{11}-D-Leu_{12}-L-Trp_{13}-D-Leu_{14}-L-Trp_{15}-ethanolamine (Sarges & Witkop, 1965a). Gramicidin B has L-phenylalanine at position 11 and gramicidin C has L-tyrosine at position 11. These three peptides and their respective variants having L-isoleucine-1 in place of L-valine-1 can be separated by countercurrent distribution (Ramachandran, 1963) or by reversed-phase liquid chromatography (Koeppe & Weiss, 1981).

We now describe a new example of heterogeneity among naturally produced linear gramicidins: peptides that are larger and more hydrophobic than the previously described gramicidins. These new peptides as a group are designated gramicidin K. Two components of gramicidin K can be resolved: gramicidin K-C, which contains tyrosine, and gramicidin K-A, which does not. Like the familiar linear gramicidins A, B, and C, gramicidins K-C and K-A form cation channels having well-defined and distinctive conductances and lifetimes in lipid bilayer membranes. Gramicidin K was discovered in the course of chromatographic purification of gramicidins A, B, and C from commercially available mixtures, where we observed a compound that was strongly retained on reversed-

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phase phenyl silica columns. We report here initial characterizations of structural and single-channel properties of this compound, gramicidin K.

Experimental Procedures

Materials. Gramicidin was purchased from ICN Life Sciences Group, from Sigma Chemical Co., and from U.S. Biochemical Corp. Chromosorb LC-5, a 37–44 μM phenyl reversed-phase pellicular packing, was a product of Johns-Manville Corp. High-pressure liquid chromatographic (HPLC) grade methanol was from either Baker or Fisher. A prepacked column of Zorbax C8, a 6 μM octyl reversed-phase spherical packing, was obtained from Du Pont Instruments. Constant-boiling HCl was from Pierce Chemical Co. Water was deionized and glass distilled. All other chemicals were reagent grade.

Methods. Chromosorb LC-5 was packed by hand as a dry powder into one 0.45 × 100 cm stainless steel column and four 0.78 × 60 cm columns. The columns were agitated to ensure complete filling with the powder and then joined in series to form a single long column. Methanol was slowly pumped into the column, and the packing was washed with a minimum of 10 L of methanol before the first use.

The above preparative column was equilibrated at room temperature with 70% methanol (30% water). A 300-mg sample of commercial gramicidin was applied to the column and eluted at 0.45 mL/min over a period of 72 h by using a complex gradient scheme: 45–50 h of isocratic elution at 70% methanol, then a 1-h gradient to 76% methanol, 10 h at 76% methanol, a 1-h gradient to 85% methanol, and finally 15 h at 85% methanol. A Spectra-Physics 8700 solvent delivery system was used to mix and pump the solvents. Gramicidins in the effluent were detected by absorbance at 280 nm and verified by analysis on the Zorbax C8 HPLC column with either 83% methanol [Figure 1 of Hinton & Koeppe (1985)] or 90% methanol (see Figure 4) as the eluting solvent.

The molar absorbance of gramicidin K was determined by dissolving an accurately weighed, vacuum-dried 5-mg sample in methanol and recording the UV spectrum with a Cary 210 spectrophotometer. The UV spectra of gramicidins A and K are indistinguishable between 250 and 330 nm, and ϵ_{282} is 21 000 M^{-1} for both compounds.

For amino acid analysis 30-μg samples of gramicidins A and K were delivered in methanolic solutions to glass tubes. Following evaporation of the methanol and thorough vacuum drying, the samples were hydrolyzed for periods of 12–96 h in constant-boiling HCl, at 110 °C in vacuo. In some experiments arginine, glutamic acid, and/or phenylalanine were included as internal standards. Aliquots (3 nmol, 5–7 μg) of the hydrolysates were analyzed on a microprocessor-controlled microbore amino acid analyzer designed at the University of Arkansas (Durham & Geren, 1981).

The molecular weight of gramicidin K was estimated by two independent methods: quantitative amino acid analysis, using internal standards to determine absolute recoveries, and gel permeation chromatography. The latter was carried out on two 1.5 × 100 cm columns of Sephadex LH-20 (Pharmacia Fine Chemicals) in series, pumped at 1 mL/min (ascending, valve injection of the sample).

NMR spectra were recorded with a Jeol FX-90Q Fourier-transform NMR spectrometer. Samples (8–10 mg) of gramicidin K or A in 50 μL of dimethyl-d_6 sulfoxide in sealed glass capillary tubes were pulsed for a minimum of 400 (^{1}H) or 30 000 (^{13}C) transients. The ^{13}C scans were restricted to a 500-Hz region that spanned the carbonyl region of the spectrum.

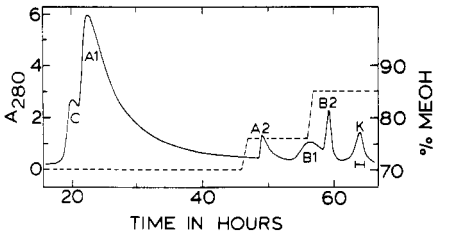

FIGURE 1: Reversed-phase chromatographic resolution of gramicidins C, A, B, and K. Component A2 is enriched in Ile^1-gramicidin A. The column of Chromosorb LC-5 was packed as a 0.45 × 100 cm section followed by a 0.78 × 240 cm section. A 300-mg sample of commercial gramicidin was applied to the column and eluted with the indicated water–methanol gradient at 0.45 mL/min and room temperature. The gramicidin K peak was pooled as indicated.

Single-channel measurements in planar bilayer membranes of diphytanoylphosphatidylcholine were made in the laboratory of Professor Olaf S. Andersen (Andersen, 1983).

Results

Figure 1 shows a previously unreported gramicidin that eluates after gramicidin B on a preparative reversed-phase chromatographic column. The proportion of this new gramicidin, as well as of gramicidin B, in the mixture varies from one batch to another. For the sample from U.S. Biochemical Corp. depicted in Figure 1, gramicidin K is about 4% of the total. Other batches have contained less than 0.5% gramicidin K. Because gramicidins A, B, C, D, M, and S had already appeared in the literature, we chose a new letter, gramicidin K, to represent the material under the new peak.

The molecular weight of gramicidin K is 2300 ± 130 by gel permeation chromatography on Sephadex LH-20 using gramicidin A, malonylbis(deformylgramicidin A) (Urry et al., 1971; Bamberg & Janko, 1977), and *N*-acetyltryptophanamide as reference compounds. An independent estimate of molecular weight by quantitative amino acid analysis indicates 2 mol of alanine and 4 mol of leucine per 2250 ± 150 g of gramicidin K. Thus the observed molecular weight of gramicidin K exceeds that of gramicidin A (1879) by approximately 400, while it is considerably smaller than the molecular weight of a dimer of gramicidin A.

As summarized in Table I, gramicidin K has the same amino acid composition as a mixture of gramicidins A and C. Thus it appears that the larger size of gramicidin K is due to non amino acid components. A formyl group was demonstrated by the chromatropic acid test (Sarges & Witkop, 1965b), and a glycine:formyl ratio of 1:1 was demonstrated by ^{13}C NMR spectroscopy (Figure 2). As has been known for gramicidin A (Hotchkiss, 1941), the tryptophans of gramicidin K are remarkably stable to hydrolysis in 6 N HCl. We recovered up to 3.38 Trp residues per mole of gramicidin K after a 96-h hydrolysis. The qualitative and quantitative similarities of the UV spectra (same peaks at 274, 282, and 290 nm and same ϵ_{282}) indicate that gramicidins A and K probably have the same tryptophan content. Furthermore, the relative areas under the indole N–H (11 ppm) and aromatic C–H (7–9 ppm) resonances in the ^{1}H NMR spectra (Figure 3) of gramicidins A and K are the same, not only in proportion to each other but also to other proton resonances. Thus, three independent methods—amino acid analysis, UV spectroscopy, and NMR spectroscopy—all indicate that the major component of gramicidin K has 4 Trp residues per mole, as does gramicidin A.

The observed nonstoichiometric amounts of tyrosine and isoleucine probably reflect sequence heterogeneity within the gramicidin K population similar to that within the gramicidin

Table I: Amino Acid Composition of Gramicidin K[a]

	obsd	mol/2300-g sample	% D[b]	% L[c]
formyl	0.95[d]	1		
Gly	1.03[e]	1		
Ala	2.00[e]	2	0	100
Val	4.05[f]	4	50	50
Leu	3.97[f]	4	84	0
Trp	3.38[f]	4 (K-A) 3 (K-C)	0	100
ethanolamine	0.94[g]	1		
Tyr	0.28[f]	0 (K-A) 1 (K-C)	0	100
Ile	0.09[f]	0.1	0	100
Phe	0.00[f]	0.0		

[a] The data are shown for a mixture consisting of 30% gramicidin K-C and 70% gramicidin K-A (see Figure 4). The individual pure components were also analyzed and found to differ only in their content of tyrosine and tryptophan. All values except Trp represent the average of at least three separate determinations. The value of 3.38 for Trp is the highest observed in any hydrolysis; 3.7 is the expected maximum yield of Trp based on NMR and UV data (see text). [b] Oxidized by D-amino-acid oxidase. [c] Oxidized by L-amino-acid oxidase. [d] Determined by ^{13}C NMR spectroscopy (Figure 2) as a ratio to the known amount of Gly. [e] Average value from 12-, 24-, and 48-h hydrolyses using Arg, Glu, and/or Phe as internal standards to determine absolute recoveries and molecular weights. [f] Determined from 96-h hydrolysis as a ratio to the known amount of Ala. [g] Determined from 12-h hydrolysis.

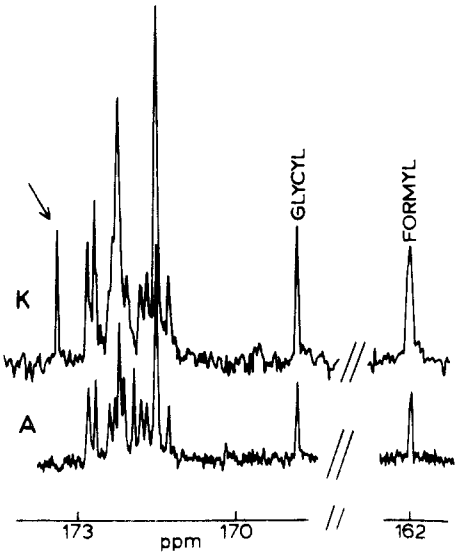

FIGURE 2: ^{13}C NMR spectra of carbonyl carbon regions for gramicidins A and K in dimethyl-d_6 sulfoxide. In both cases the glycine:formyl stoichiometry is 1:1. The arrow designates a new or shifted carbonyl carbon resonance of gramicidin K.

A family. On a high-resolution reversed-phase octyl silica column, gramicidin K can be resolved into two components (Figure 4). Under the conditions normally used for eluting gramicidin A from such a column (ca. 80% methanol), gramicidin K remains bound to the column indefinitely. In 90% methanol, however, two components of gramicidin K can be eluted from the column: an initial peak representing 30% of the material and containing 1 equiv of tyrosine and a second peak representing 70% of the material and containing no tyrosine. The fractional amount of tyrosine in Table I is thus explained. Note that the two components elute in the same relative order as gramicidins C and A, which also differ in tyrosine content, although both of the gramicidin K components are much more strongly retained by the column. By analogy with gramicidins C and A, we designate the minor tyrosine-containing component gramicidin K-C and the major component gramicidin K-A.

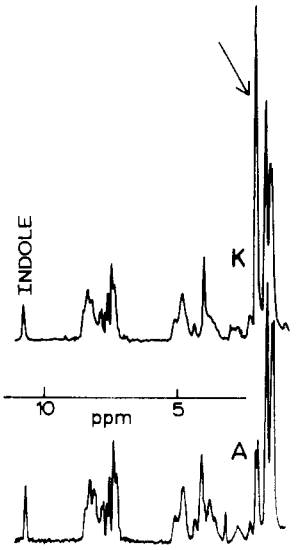

FIGURE 3: ^1H NMR spectra of gramicidins A and K in dimethyl-d_6 sulfoxide. The peak designated by the arrow is characteristic of $-CH_2-$ groups and indicates about 20–25 additional ^1H nuclei in gramicidin K.

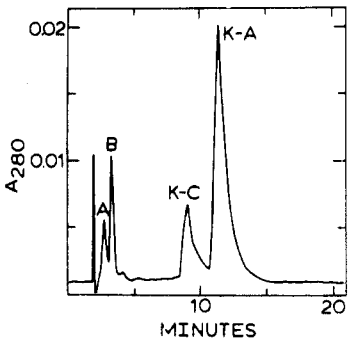

FIGURE 4: Resolution of components K-C and K-A from gramicidin K. A 2-μg aliquot from the gramicidin K peak of Figure 1 was rechromatographed on a 0.46 × 25 cm column of Zorbax C8 in 90% methanol–10% water at 1.5 mL/min, 1800 psi, and room temperature. The K-C and K-A components were collected as indicated, diluted with ethanol, and used directly for single-channel experiments. Trace amounts of gramicidins A and B have been included as reference markers in the chromatogram.

The amino acid sequence is not yet known, but gramicidin K-A appears to have the amino acid composition of gramicidin A, together with some additional chemical moieties that comprise some 400 daltons and confer additional hydrophobicity upon the peptide. A possible indication of the nature of an extra component of gramicidin K comes from ^1H NMR spectroscopy. *The peak representing $-CH_2-$ groups in the ^1H NMR spectrum (Glickson et al., 1972) is 3-fold larger for gramicidin K than for gramicidin A (Figure 3).* The increase in the area under this aliphatic ^1H NMR peak represents about 20–25 protons. The remainder of the ^1H NMR spectrum of gramicidin K is similar to that of gramicidin A. It thus appears likely that gramicidin K consists of gramicidin A plus an attached aliphatic lipid-like group. This composition would be consistent with the chromatographic behavior of gramicidin K. The likely attachment site would be the ethanolamine group. There could not be anything attached at the formyl end of the molecule because of the characteristic [^{13}C]carbonyl chemical shift for the formyl carbon (Figure 2).

A new [^{13}C]carbonyl peak for gramicidin K is evident in Figure 2 to the left of the large cluster of carbonyl peaks. This peak could represent either a new ester or an amide carbonyl group not present in gramicidin A or, alternatively, a dra-

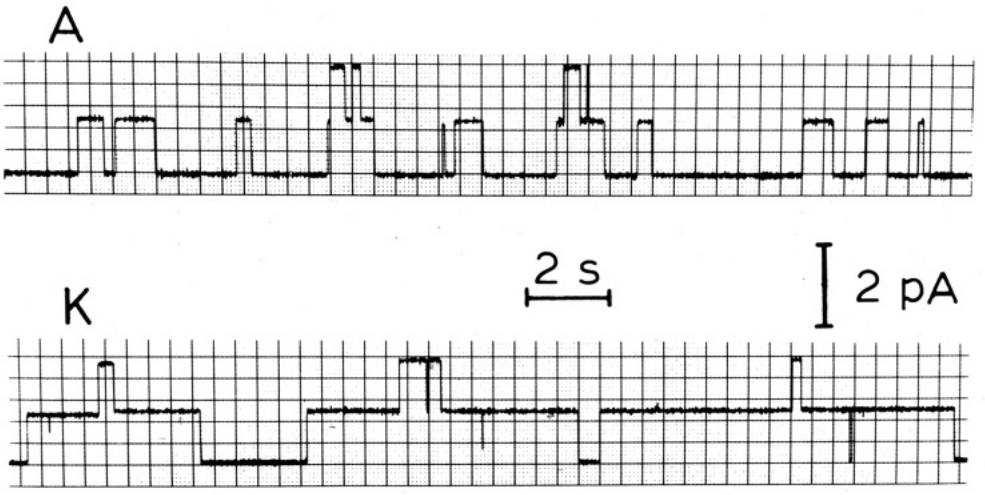

FIGURE 5: Single-channel data for valine-gramicidin K (lower) compared to valine-gramicidin A (upper). The single-channel measurements were done as described elsewhere (Andersen, 1983). The membrane-forming solution was diphytanoylphosphatidylcholine–*n*-decane (2.5% w/v); the aqueous phase was unbuffered 1.0 M NaCl at 100 mV and 25 °C. Calibration bars denote 2 pA and 2 s, respectively. The upper gramicidin A record contains 15 channel openings with a total duration of 7.5 s. The lower gramicidin K record contains eight (or nine) channel openings with a total duration of 20 s.

matically different environment for one of the carbonyl carbons of gramicidin A. An ester bond between the ethanolamine –OH and a fatty acid is the most likely possibility.

The gramicidin K-C and K-A components shown in Figure 4 were collected and tested for single-channel properties in planar bilayer membranes. In the presence of 1.0 M NaCl and 100-mV applied potential, gramicidins K-A and K-C give rise to unique and reproducible single-channel events (Figure 5). The respective conductances of gramicidin K-A and K-C channels are about 10% lower than those of gramicidin A and C channels (Whaley et al., 1985). As is evident from Figure 5, the lifetimes of the K-type channels are 3–5 times longer. A detailed analysis of the properties of these channels will be the subject of a future paper (O. S. Andersen et al., unpublished results).

Discussion

This paper describes new cation-specific channels that appear to be lipopeptide derivatives of gramicidins A and C. The presumed structures of the gramicidin K species, consisting of a fatty acid or other hydrophobic group esterified to the ethanolamine of gramicidin A or C, lead to a prediction that mild base hydrolysis could perhaps produce gramicidins A and/or C from gramicidin K. Indeed, we have recently demonstrated that treatment of gramicidin K with mild methanolic NaOH yields a mixture of compounds that behave on HPLC like Val^1- and Ile^1-gramicidins A and C (R. E. Koeppe II and R. Corder, unpublished experiments). However, alternate sequences may migrate similarly on the HPLC column, and the structural assignment must be regarded as tentative at this time.

A notable consequence of the hydrophobic component of the gramicidin K's is the longer mean lifetime for the channels. Channel lifetime presumably reflects the stability of a conducting gramicidin dimer in a membrane (Hladky & Haydon, 1972; Morrow et al., 1979; Szabo & Urry, 1979; Bradley et al., 1981; Elliott et al., 1983). The hydrophobic component apparently makes the conducting gramicidin K dimer more stable and increases its lifetime.

Molecular models of the gramicidin A channel generally represent the ethanolamine –OH group in a hydrogen bond to the carbonyl oxygen of Trp-11 (Venkatachalam & Urry, 1983; Koeppe & Kimura, 1984; Etchebest & Pullman, 1984). Obviously, such a hydrogen bond would not be possible for a derivatized ethanolamine, as we propose for gramicidin K. Recent calculations have suggested that the energy profiles for alkali-metal cations moving through the channel are modified notably by allowing for conformational flexibility of the ethanolamine end (Etchebest et al., 1984). In this regard, it is noteworthy that the gramicidin K conductance is comparable to the gramicidin A conductance. Gramicidin K may provide opportunities for further experimental and theoretical tests of the role of the ethanolamine end of the channel in ion binding and transport.

In spite of its low relative abundance, the different conductance and lifetime of gramicidin K channels (and of gramicidin B channels) argue against using unfractionated mixtures of commercial gramicidins. However, as most investigators use chromatographically purified gramicidins for single-channel work, the presence of gramicidin K in commercial mixtures is unlikely to contribute to a dispersity of single-channel currents [e.g., see Busath & Szabo (1981) and Urry et al. (1984)]. Furthermore, the gramicidin channels that exhibit variant conductances have been found to have lifetimes equal to or shorter than typical gramicidin A channels (Busath & Szabo, 1981), whereas gramicidin K channels have longer lifetimes. The variable yields of gramicidin K in different batches are probably due to variations in commercial procedures for extracting and fractionating gramicidins. Because of its highly nonpolar character, gramicidin K could easily be totally or partially removed from some batches and not others. Thus it is possible that the relative abundance of gramicidin K in crude extracts may actually exceed 4% of total gramicidin.

The in vivo presence of gramicidin K has not yet been demonstrated. Gramicidin K could function as a biosynthetic precursor of gramicidin A or, alternatively, could be synthesized from gramicidin A, either for a specific function or perhaps as the first step in a degradation scheme. The biosynthesis of the C-terminal portion of gramicidin A is not well understood. Two enzymes responsible for the biosynthesis of the initial heptapeptide of gramicidin A have been described (Akers et al., 1977). These enzymes contain a 4′-phosphopantetheine cofactor and carry out the ATP-dependent activation, site-specific racemization, and sequential addition of L-Val, Gly, L-Ala, and L-Leu to form L-Val-Gly-L-Ala-D-Leu-L-Ala-D-Val-L-Val. Formylation occurs sometime after chain initiation by an unknown mechanism. The remaining

portions of the gramicidin peptides are presumably synthesized in similar fashion to the initial heptapeptide sequence, but the enzymes responsible for the later stages of gramicidin biosynthesis have not been described. Gramicidin K may prove to be an important tool for deciphering the mechanisms involved in synthesizing the linear gramicidins.

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