Biochimica et Biophysica Acta, 982 (1989) 223–227
Elsevier

BBAMEM 74460

Synthesis and channel properties of [Tau^{16}]gramicidin A

Roger W. Roeske [1], Tanya P. Hrinyo-Pavlina [1], Richard S. Pottorf [1,*], Terry Bridal [2], Xian-Zheng Jin [2] and David Busath [2]

[1] *Department of Biochemistry, Indiana University School of Medicine, Indianapolis, IN and* [2] *Division of Biology and Medicine, Brown University, Providence, RI (U.S.A.)*

(Received 6 October 1988)
(Revised manuscript received 13 March 1989)

Key words: Des(ethanolamine)-taurine^{16}-gramicidin A; Gramicidin A, [Tau^{16}]-; Single-channel conductance; Ion channel; Planar membrane

Des(ethanolamine)-taurine^{16}-gramicidin A ([Tau^{16}]gramicidin A) was synthesized by the solid phase method and its channel-forming behavior in planar lipid bilayers was examined. The purified monovalent anionic peptide formed channels when applied to the aqueous compartments on both sides of the bilayer, but not when applied to one side only. The single-channel conductance was measured for KCl concentrations between 0.1 and 1.0 M and was found to be higher than that of gramicidin A in each case. Single-channel lifetimes were similar to those of gramicidin A suggesting that the channels have the $\beta^{6,3}$ helix structure.

Introduction

Protein channels in cell membranes are thought to have charged groups near the ion transport pathway [1–3] which can be titrated [4–6] or modified by specific reagents [7,8]. The impact of fixed charges in the channel wall on channel conductance and selectivity has been explored for the excitable sodium channel [7] and the acetylcholine (ACh) receptor channel [9]. The amino acid sequences for many channels are known, but the detailed atomic structures of these channels are as yet poorly established.

Model channels formed by analogs of gramicidin A (Fig. 1) provide the opportunity to examine the role of fixed charges where the atomic structure of the peptide walls is well established. In lipid bilayers, gramicidin forms cation-selective channels comprised of neutral head-to-head dimers of $\beta^{6,3}$-helices (for a review, see Ref. 10). Charged C-terminus analogs produce channels with the charged atoms imbedded in the channel wall at both the entry and exit.

Two charged analogs have been produced and studied previously. *O*-pyromellityl gramicidin [11] has three

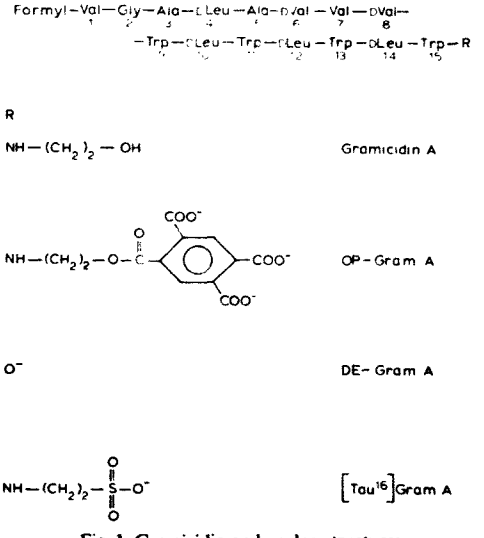

Fig. 1. Gramicidin and analog structures.

* Present address: Experimental Station, E.I. duPont Corp., Wilmington, DE 19898, U.S.A.

Abbreviations: Z, benzyloxycarbonyl; TFA, trifluoroacetic acid; CMA, chloroform/methanol/acetic acid (85:15:3, v/v); MBHA, methylbenzhydrylamine; Boc, *tert*-butyloxycarbonyl; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole; DIEA, diisopropylethylamine; NMP, *N*-methylpyrrolidinone; OP-Gram, *O*-pyromellityl gramicidin; DE-Gram, desethanolamine gramicidin A; [Tau^{16}]gramicidin A, des(ethanolamine)-taurine^{16}-gramicidin A; TFE, polytetrafluoroethylene.

Correspondence: D. Busath, Box G, Brown University, Providence, RI 02912, U.S.A.

negative charges on a phenyl group (Fig. 1) located at the C-terminus of gramicidin A or gramicidin D, a commercially available mixture of gramicidins A, B, and C [12]. The charged group might be poorly localized near the channel mouth because of its bulk. Apell et al. [11] observed that it formed channels which had lifetimes and conductance properties which were similar to those of gramicidin A except that at low saline concentrations the single-channel conductance was higher. They also observed that channels would only form if the peptide was added to the bathing solutions on both sides of the lipid membrane. They interpreted these differences to mean that (a) the charged group attracted permeant cations (K^+) to the channel mouth but the effect was diminished at high ionic strength by shielding of the charged group; and (b) the charge on the peptide anchors its carboxyl terminus to one side of the bilayer. The trivalent O-pyromellityl group is well-designed to anchor the peptide, but it may be susceptible to hydrolysis and the group's bulkiness produces a complicated geometry at the channel entrance making it difficult to predict its effect on channel conductance.

Desethanolamine gramicidin A [13] has a simpler geometry than OP-Gram, but paradoxically yielded two distinct populations of long-lived channels at all pH values when incorporated into lipid bilayers. Only one channel conductance level was expected because, even though the channels would have two titratable oxygens at each end of the channel, charges should fluctuate at frequencies too high to be detectable in the single-channel currents. The authors deemed it unlikely that one channel type was due to an impurity in peptide composition because the peptide had been highly purified using HPLC. It was postulated that the two conductance populations may represent two different stable peptide conformations.

In an effort to produce a more stable, permanently charged peptide and hoping to avoid the ambiguity due to the dual population observed with DE-Gram, we have synthesized [Tau[16]]gramicidin. The molecule is very stable in aqueous solution, having only amide bonds. It has a low pK; the pK for the sulfonic acid in free taurine is 1.5, and is probably slightly higher in [Tau[16]]gramicidin. Thus the molecule is almost totally deprotonated under normal conditions. The negative charge should be shared equally by the three oxygens due to resonance of the double bonds. We report here the details of the peptide synthesis and our initial observations of the properties of symmetric [Tau[16]] gramicidin A channels. So far as we are aware, this is the first synthesis of a C-terminal sulfonic acid peptide by the solid phase method. The phenyl ester link to the polystyrene support is well suited for sulfonic acids, being very stable to the acidic conditions encountered during synthesis yet easily cleaved by aqueous base to remove the completed peptide from the resin.

Materials and Methods

Peptide synthesis

The phenyl ester link between the C-terminal taurine residue and the polypeptide support was prepared as follows. Taurine (5.0 g) was carbobenzoxylated according to the standard procedure [14] to give a mixture of Z-taurine and KCl. After thorough drying, the mixture was refluxed with 60 ml of thionyl chloride for 3 h. Removal of the thionyl chloride by vacuum distillation gave a residue of 7.6 g, consisting of Z-taurine chloride and salts. This was mixed immediately with 2.35 g of *tert*-butyl-*p*-hydroxybenzoate in 250 ml pyridine and stirred at room temperature overnight. Evaporation of the pyridine left a dark red syrup which was dissolved in EtOAc, washed with 0.1 M HCl, 7 × 70 ml, and with water. After drying, the EtOAc was distilled to give 4.3 g of a red-brown solid which was purified by flash chromatography on a 4.5 × 16 cm column of silica gel, eluting with a mixture of 99.5 parts of $CHCl_3$ and 0.5 parts of MeOH. White crystals were obtained from $CHCl_3$ petroleum ether, 2.8 g (51% yield), m.p. 109–110 °C, tlc: R_f 0.82 (CMA). Treatment of the *tert*-butyl ester with 15 ml of 50% trifluoroacetic acid in $CHCl_3$ for 1 h gave, after recrystallization from EtOAc/petroleum ether, 2.0 g (82%) of Z-taurine *p*-carboxy phenyl ester, m.p. 155–156 °C, tlc: R_f 0.58 (CMA 85 : 15 : 3). This was attached, using DCC + HOBt, to 6.2 g of 1% cross-linked MBHA polystyrene resin to which had been added one residue of phenylalanine, 0.2 mequiv/g. The Z group was removed by treatment of the resin with 50 ml 4 M HBr in HOAc for 60 min, after which the resin was transferred to the reaction vessel of a Beckman 990B automated peptide synthesizer. The first two residues, Boc-L-Trp and Boc-D-Leu, were coupled by the symmetrical anhydride method, using a 6-fold excess, and the chain was then elongated by successive coupling with 3-fold excess of Boc amino acids using DCC + HOBt. Boc protecting groups were removed in each cycle by treatment for 30 min with 50% TFA in $CHCl_3$ containing 7% thioanisole. After removal of the final Boc group and neutralization with DIEA, the peptide was formylated with *p*-nitrophenyl formate, 20-fold excess, for 16 h.

To effect cleavage of peptide from the resin, a 300 mg portion of the peptide-resin was suspended in 3 ml of NMP containing 0.25 ml of 2 M KOH and stirred at room temperature for 15 h. The resin was filtered and washed with 10 ml of NMP. Addition of 150 ml of ether to the combined NMP solutions precipitated a gummy residue, which was taken up in MeOH. The methanol solution was concentrated, diluted with water and lyophilized to give 130 mg of a white powder (peptide + KOH). This crude peptide was purified by preparative reversed phase chromatography on C_{18} silica gel [15],

eluted with 73% aqueous MeOH, 0.1% TFA, to give pure [Tau[16]]gramicidin in an overall yield of 11%.

For amino acid analysis, a sample hydrolyzed in methanesulfonic acid [16] gave satisfactory ratios of all the amino acids except valine, which was 12% low. Hydrolysis in 6 M HCl for 40 h destroyed tryptophan but gave good ratios of all other residues, including valine. Similar results have been observed with another analog of gramicidin A [17].

FAB mass spectrum (Xe; matrix thioglycerol), m/z 1946.05; Calc. (M + H) 1946.0403. Major peaks were also seen at 1968 (M + Na) and at 1984 (M + K).

Bilayer conductance

Measurements of lipid membrane conductance induced by [Tau[16]]gramicidin were made at two peptide concentrations. High concentrations yielded membranes with many channels which were used to examine whether the negative charge on the analog prevents it from crossing the lipid bilayer. At low concentrations, single-channel measurements were made on [Tau[16]] gramicidin to compare with those of normal gramicidin channels.

Multi-channel membrane conductance measurements. Planar lipid bilayer membranes used to make conductance measurements were formed from a solution of glycerol monoolein (NuChek Prep, Elysian, MN) in hexadecane (50 mg/ml). Membranes were formed across a partition aperture 830 μm in diameter (0.54 mm^2) which joined two reservoirs machined in a solid TFE block. Each reservoir was filled with 2.0 ml unbuffered aqueous 1 M KCl (pH = 5.6) and was constantly magnet-stirred. A 100 mV potential was applied via Ag-AgCl wire electrodes. Changes in the transmembrane ion current were monitored with a current-to-voltage amplifier having a feedback resistance of 100 kΩ. The signal was low-pass filtered, sampled 10 times per second and stored digitally. Measurements were made at room temperature, 25–27°C.

After membrane formation, the conductance was monitored for 2 min in the absence of gramicidin. Then, 2 μl of 0.1 μg/ml [Tau[16]]gramicidin in methanol was added to the same reservoir that contained the electrode where the +100 mV potential was applied (*cis* reservoir), and membrane conductance measured for 5 min. At that point, an additional 2 μl of 0.1 μg/ml peptide solution was added to the virtual ground (*trans*) reservoir and the conductance measured for another 5 min. Finally, the membrane was broken by applying 1.0 V. The membrane resistance always exceeded the series resistance (electrodes and membrane-free aperture) by greater than 10-fold.

Single-channel current measurements. For single-channel current measurements, glyceryl monoolein/hexadecane membranes were formed across an 80 μm aperture fashioned on a PE pipette that comprised one compartment of the chamber used. The pipette was melted and shaved at the tip to the specified aperture size, mitred, and press-fit into a TFE block containing a 3.0 ml reservoir. The block reservoir was filled with 1 M KCl solution and a small volume drawn into the pipette by gentle suction to adequately bathe a Ag-AgCl electrode suspended in the pipette. A +100 mV potential was applied to this electrode while the block reservoir was held at virtual ground with a second Ag-AgCl electrode. 2 μl of 2.2 ng/ml [Tau[16]]gramicidin in methanol was added to the block reservoir before membrane formation. The current-to-voltage converter had a feedback resistance of 10 GΩ. The signal was filtered and sampled continuously 100 times per s for 6-min periods. Only the channels whose conductance fell within approx. 3 S.D. of the gaussian peak mean were included in the calculations of main peak single channel conductance and lifetime. Single-channel measurements were made at room temperature, usually 23–24°C. When the temperature was outside of that range, single-channel conductances were corrected to 24°C using $Q_{20} = 1.9$ [18].

Results

Fig. 2 shows membrane conductance as a function of time for a multi-channel experiment. At arrow *a*, [Tau[16]]gramicidin A is added to the *cis* chamber. After 5 mins, there had been little increase in membrane conductance. At arrow *b*, addition of [Tau[16]]gramicidin A to the *trans* side caused a rapid increase in membrane current. In four experiments, *cis* [Tau[16]]gramicidin placement yielded a slight increase in mean membrane conductance (0.16 μS ± 0.32 μS, S.D.) above the

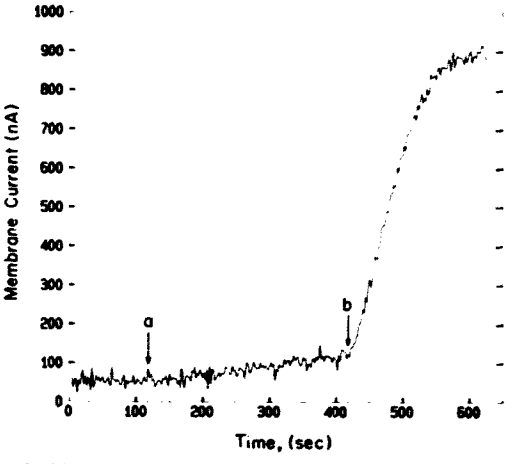

Fig. 2. Multichannel membrane current with no peptide present initially, after addition of [Tau[16]]gramicidin A to the *cis* chamber (final concentration: 0.1 ng/ml) at point *a*, and addition of [Tau[16]]gramicidin A (to 0.1 ng/ml) to the *trans* chamber at point *b* 1.0 M KCl. R_s, 5–10 kΩ. V_m, 100 mV. 26°C.

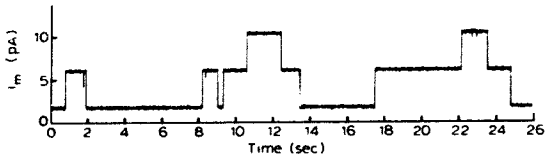

Fig. 3. Transmembrane current jumps produced by addition of [Tau^{16}]gramicidin A to both compartments to yield a peptide concentration of 2.2 pg/ml. 1.0 M KCl. V_m, 100 mV. 23° C.

peptide-free membrane conductance. Subsequent addition of peptide to the *trans* side yielded a large conductance increase averaging 8.26 μS ($\pm$2.39 μS S.D.).

The single-channel current transitions produced by [Tau^{16}]gramicidin A are similar to those produced by gramicidin A. Fig. 3 is a membrane current trace showing the usual all-or-nothing channel conductance pattern. Unlike DE-Gram [13] which produced two distinct peaks in the single-channel conductance histogram, [Tau^{16}]gramicidin channels formed only one main peak. This is demonstrated in Fig. 4 which is a histogram of single-channel currents composed using data collected from 15 membranes. The main peak (between 43.0 and 47.0 pS) has a mean of 4.48 pA, a standard deviation of 0.07 pA, and contains approx. 68% of the channels observed during the experiments. The lifetimes of channels from the main peak had an average duration of 3.59 s ($\pm$0.19 s, S.E., n = 382). These characteristics are similar to those observed for channels formed by gramicidin A [19].

At reduced [KCl], the single-channel conductances were higher than those of gramicidin A as shown in Table I. The first and second columns give the single-channel conductance and the ratio of [Tau^{16}]gramicidin

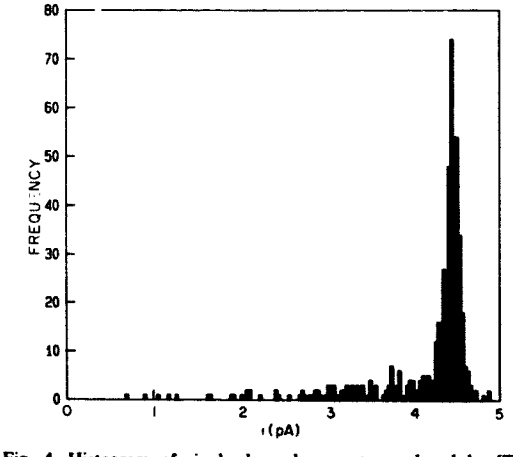

Fig. 4. Histogram of single-channel currents produced by [Tau^{16}] gramicidin A (1.1 pg/ml). 15 membranes composite. Peak mean current, 4.48 $\pm$ 0.07 pA. (361 channels of 529 observed: 68% main peak, 32% mini channels). Peak mean lifetime, 3.59 s $\pm$ 3.66 s (S.D.). 1.0 M KCl. V_m, 100 mV. 23° C.

TABLE I

Single-channel conductance ratio (charged / neutral peptide)

[Cation] (M)	[Tau^{16}]-Gramicidin A [a]		OP-Gram [b] ratio	DE-Gram [c] ratio
	γ (pS)	ratio		
0.1	27.7	2.25	2.20	4.16
0.2	35.8	1.74	1.65	
0.5	44.5	1.34	1.01	
1.0	46.8	1.02	0.80	1.59

[a] For [Tau^{16}]gramicidin A, single-channel conductance, γ, is the mean of three or four measurements using KCl at 100 mV (S.D. between 1.0 and 3.5 pS) and the ratio was calculated using the single channel KCl conductances reported for gramicidin A at 50 mV by Neher et al. [20]. [b] OP-Gram ratios were taken from Fig. 11 in Apell et al. [11] where the neutral peptide was gramicidin A and conductance was measured using CsCl at 50 mV. [c] DE-Gram A ratios were taken from Fig. 6 of Reinhardt et al. [13] where the conductance of the major channel type was measured using CsCl at 50 mV at pH 6.8 ('charged') and 3.2 ('neutral') (pK measured as 3.82 using the pH dependence of the single-channel conductance).

A conductance (our measurement) to gramicidin A conductance [19] for different KCl concentrations. The ratios are similar to those obtained for the trivalent anionic OP-Gram at different Cs^+ concentrations [11] shown in column 3. The fourth column gives the ratio of DE-Gram cesium conductances at two different pH values where the peptide is mostly monovalent anionic or mostly neutral due to protonation of its C-terminus [13]. The conductance enhancement, presumed to be due to the charge at the channel mouth. is greater for DE-Gram than for the other two peptides. (This effect was also evident at other intermediary concentrations not included in Table I). For all three peptides, the enhancement decreased with increasing bath ionic strength, consistent with the hypothesis that the anion charge at the mouth of the channel can be shielded by the bath solution [11].

Discussion

[Tau^{16}]Gramicidin differs from gramicidin in that it has a fixed negative charge at the carboxyl terminus, the channel mouth. This negative charge is expected to function like that in OP-Gram [11]: (a) it should anchor the molecule on one side of the membrane, and (b) enhance the cation concentration near the channel mouth, thus enhancing channel conductance when ion entry limits the rate of ion transport. Furthermore, it has the advantage over OP-Gram in that it is simpler in structure and more chemically stable.

The above results indicate that [Tau^{16}]gramicidin remains on one side of the membrane when added to the *cis* side. The slight conductance increase observed when the peptide was added only to the *cis* side was not very reproducible. In three of the four trials whose average increase was reported above, there was no dis-

tinguishable increase. The one case where a small increase was observed (the experiment depicted in Fig. 2) may have been due to slight contamination of the *trans* chamber with peptide.

OP-Gram channels have lifetimes which are similar in average duration to those of gramicidin in monoolein/hexadecane bilayers [11], indicating that the secondary and quaternary structures of the channels are similar. [Tau^{16}]gramicidin channels also have similar durations, indicating that they, too, are head-to-head dimers of $\beta^{6.3}$ helices.

Apell et al. [11] found the conductance of OP-Gram A to be higher than that of gramicidin A at low ionic strengths. A similar finding was reported by Reinhardt et al. [13] for both types of channels formed by DE-Gram. In contrast to DE-Gram, [Tau^{16}]gramicidin produced only one peak in the conductance histogram. As expected, the differential between gramicidin and [Tau^{16}]gramicidin conductance increased at lower ionic strengths (Table I). The increase is not as great as that produced by the pH effect for DE-Gram. This suggests that the taurine is less effective than the carboxylic acid in enhancing the permeant cation concentration in the region of the channel entrance. This might be expected because the negative charge is distributed between three oxygen atoms in taurine rather than between only two as in the carboxylic acid. Taurine and carboxyl both enhance conductance more than O-pyromellityl, whose three negative charges are quite dispersed on the phenyl residue.

Acknowledgements

The work done at Indiana University was supported by the Grace M. Showalter Trust. We would like to thank Mr. Patrick J. Edwards for technical assistance with the synthesis and Dr. John Occolowitz of the Lilly Research Laboratories for the FAB mass spectrum. The work done at Brown University was supported by NIH (GM33361 and NS01085).

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