Large unselective pore in lipid bilayer membrane formed by positively charged peptides containing a sequence of gramicidin A

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Abstract **Ion-channel activity of a series of gramicidin A analogues carrying charged amino-acid sequences on the C-terminus of the peptide was studied on planar bilayer lipid membranes and liposomes. It was found that the analogue with the positively charged sequence GSGRRRRSQS forms classical cationic pores at low concentrations and large unselective pores at high concentrations. The peptide was predominantly in the right-handed $\beta^{6.3}$-helical conformation in liposomes as shown by circular dichroism spectroscopy. The single-channel conductance of the large pore was estimated to be 320 pS in 100 mM choline chloride as judged from the fluctuation analysis of the multi-channel current. The analogue with the negatively charged sequence GSGEEEESQS exhibited solely classical cationic channel activity. The ability of a peptide to form different type of channels can be used in the search for broad-spectrum antibiotics.**

Keywords: Transmembrane peptide; Phospholipid membrane; Selectivity; Magainin; Melittin; Alamethicin; α-Helix; Hydrophobic mismatch; Electrostatic interactions; Macromolecules

1. Introduction

During recent years much attention was attracted to interaction of peptides with lipid membranes, in particular, to the pore-forming activity of a great variety of peptides. Based on the results of numerous studies, at least five types of pore structures were proposed: (i) rigid intramolecular tubes formed by head-to-head dimerization of gramicidin A (gA) [1], (ii) intramolecular pores resulting from stacking together of cyclic peptides [2], (iii) intermolecular ''barrel-stave'' pores formed by bundles of α-helical rods of alamethicin [3], (iv) porin-like high-conductance intermolecular pores with a β-barrel structure [4], (v) intermolecular peptide–lipidic pores with pore walls formed both by peptide molecules and lipid headgroups [5–7]. The latter type is generally accepted now for positively charged amphipatic peptides like magainin [8], being of interest as a promising group of new antibiotics.

It is unclear whether some peptide can form pores of different structure. In the present work, we addressed this question by studying activity of gA derivatives. It was found that the gramicidin analogue with the positively charged amino acid sequence, e.g., GSGRRRRSQS, attached to the C-terminus forms classical potassium ion selective pores at low concentrations and large unselective pores at high concentrations.

2. Materials and methods

Peptides were prepared by standard solid-phase Nα-Fmoc methodology on Rink amide resin [4(2′,4′-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxy resin] using the diisopropylcarbodiimide/1-hydroxybenzotriazole coupling system. N-terminal formylation and N-terminal acetylation of peptides were conducted in the presence of N-ethyldiisopropylamine using 2-nitrophenyl formate and acetic anhydride, respectively. The peptide resins were treated with trifluoroacetic acid–ethandithiol–water (94:3:3) for 2.5 h. HPLC-purification of the samples gave pure peptides. The fidelity of the peptides was confirmed by MALDI-TOF MS.

Planar bilayer lipid membranes (BLMs) were formed from a 2% solution of diphytanoylphosphatidylcholine (DPhPC, Avanti Polar Lipids, Alabaster, AL) in *n*-decane (Merck, Darmstadt, Germany) by the brush technique on a hole in a Teflon partition separating two compartments of a cell containing aqueous buffer solutions. A cell with a 0.15-mm diameter hole was used in single-channel experiments, and one with a 0.55-mm diameter hole was used in multi-channel experiments. The bathing solution used was 1 M KCl (or 0.1 M KCl), 10 mM Tris, 10 mM MES, and 10 mM β-alanine, pH 7.0. The electrical current (*I*) was measured with an amplifier (U5-11, Moscow, Russia), digitized by a LabPC 1200 (National Instruments, Austin, TX) and analyzed using a personal computer with the help of WinWCP Strathclyde Electrophysiology Software designed by J. Dempster (University of Strathclyde, UK). A voltage of 30 mV (unless otherwise stated) was applied to BLM with Ag–AgCl electrodes placed directly into the cell. In single-channel experiments, a patch-clamp amplifier (model BC-525C, Warner Instruments, Hamden, CT) was used for current measurements.

Dye-loaded liposomes were prepared as described previously [9] by using an Avanti Mini-Extruder. The unbound carboxyfluorescein (CF) was then removed by passage through a Sephadex G-50 coarse column with a buffer solution containing 10 mM β-alanine, 0.12 M KCl, pH 4.0. Liposomes for circular dichroism (CD) spectroscopy were prepared as described in [10] except for the addition of 30% diphytanoylphosphatidylglycerol (DPhPG) to lipid solution used for the preparation DPhPC liposomes. CD spectra were measured at a peptide/lipid mole ratio of 1/40. Heat incubation was carried out at 68 °C for 8 h under argon.

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Abbreviations: BLM, bilayer lipid membrane; gA, gramicidin A; CD, circular dichroism; CF, carboxyfluorescein; DPhPC, diphytanoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPhPG, diphytanoylphosphatidylglycerol

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The CF leakage of liposomes was measured with a Hitachi F-4000 (Tokyo, Japan) fluorimeter with peak excitation and emission wavelengths of 490 and 520 nm (band-pass of both beams, 5 nm). The extent of the CF leakage (α) was calculated as follows: $\alpha\ (\%) = (F_f - F_0)/(F_{100} - F_0)$, where F_0 and F_f represent the initial and the final (steady-state) levels of fluorescence before and after the protein addition, and F_{100} is the fluorescence value after complete disruption of liposomes by addition of the detergent, LDAO (lauryldimethylamine-*N*-oxide, final concentration, 2.4% w/w). The buffer solution 100 mM KCl, 10 mM Tris, and 10 mM MES, pH 7, was used for the CF leakage studies.

The diffusion electrical potential on the liposome membranes was assayed using the potential-sensitive fluorescent dye safranine O (Aldrich), as described in [11]. Unilamellar lipid vesicles from egg yolk phosphatidylcholine (Avanti Polar Lipids) were prepared as described above in the solution of 200 mM KCl, 5 mM phosphate buffer, pH 7.0. 10 μl of this solution containing 0.2 mg liposomes was added to 1 ml of a solution of 340 mM sucrose, 10 mM Tris, and 10 mM MES, pH 7.0, containing 1 μM safranine O. The fluorescence emission of safranine O was monitored at 581 nm (excitation at 521 nm, 5-nm slits).

CD spectra were recorded at 20°C with the Jasco 500 C dichrograph in demountable cells (Hellma) with 10^{-2} cm optical path length. The spectra reported are the average of two or three scans. The spectrum of the appropriate suspension of liposomes without peptides was used as the baseline. Computer analysis of the CD spectra was made as described previously [10,12].

3. Results

It is known that an ion-conducting pore formed by gA in lipid membranes has the diameter of 4 Å [13,14], thus being impermeable for such bulky molecules as CF. In contrast to gA, the peptide P1C (gramicidin-GSGRRRRSQS-COOH) appeared to induce the leakage from CF-loaded liposomes (Fig. 1, compare curves 1 and 2). This effect took place at 1 μM of the peptide, but not at 0.01 μM. When only the C-terminal sequence (GSGRRRRSQS-COOH) was added to liposomes instead of the whole P1C peptide, no dye leakage was observed (curve 3, Fig. 1). The dose dependence of the P1C effect is shown in the inset to Fig. 1. The addition of the P1C peptide neither altered the liposome size, as judged by measurements with Malvern Autosizer IIc, nor induced destabilization of planar BLM even at high concentration (exceeding 10 μM). All of these data allow us to suggest that the P1C peptide does not cause membrane destruction, but induces the formation of large-diameter pores that are permeable to CF.

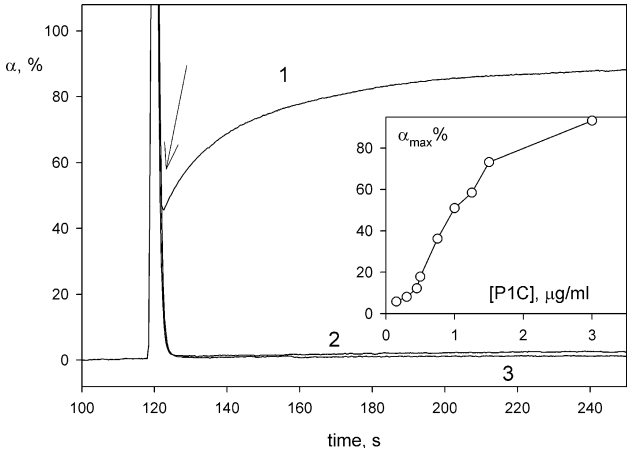

Fig. 1. The CF leakage (α denotes the extent of the leakage) from liposomes induced by P1C. P1C, 2 μg/ml, was added at the moment marked by an arrow. Curves 2 and 3 represent control responses of the CF fluorescence observed after the additions of gA and the peptide GSGRRRRSQS (the C-terminus sequence of the P1C peptide), respectively, at the same concentration as P1C. Inset shows the dependence of α on the P1C concentration.

Table 1 summarizes the data on the CF leakage from liposomes induced by gA and its different derivatives. It is seen that of the peptides tested all those having a positively charged sequence at the C-terminus efficiently induced the CF leakage. There was no marked difference in the activity between peptides containing lysine or arginine residues (compare the data obtained for P2C and P3C with those for P1C). The positively charged peptide acetylated at the N-terminus (P5C) also appeared to be a potent inducer of the CF leakage, although it is known that N-acetyl gramicidin is about two orders of magnitude less efficient than gA in formation of potassium-conducting channels [15,16]. In agreement with the literature data, our measurements showed that to obtain similar levels of the K^+ conductance of the planar BLM we had to add the two-orders of magnitude larger amount of P5C as compared to P3C (Table 1).

It is important to note that the negatively charged analogue of P1C having four glutamine residues instead of arginine residues (N1C) was inefficient in promoting the CF leakage from liposomes, though it induced potassium conductance of planar BLM (Table 1).

In further experiments we compared the potassium fluxes across lipid bilayer membranes induced by gA and its positively and negatively charged derivatives by monitoring membrane potential with the potential-sensitive fluorescent dye safranine O [11,17]. As seen from curve 1 in Fig. 2A, no fluorescence change was observed after the addition of gA to K^+-loaded liposomes in the absence of K^+ gradient across the membrane. Curve 2 shows that the addition of gA to K^+-loaded liposomes placed into the K^+-free buffer containing isotonic sucrose led to an increase in safranine fluorescence due to the development of K^+ diffusion potential across the liposome membrane. The similar result was obtained with the negatively charged analogue N1C (curve 3). By contrast, the addition of P1C did not provoke an increase, but led to some decrease in safranine fluorescence (curve 4), which could be explained by the existence of small diffusion potential provided by passive K^+ leakage through the membrane. Thus it can be concluded that large pores formed by P1C are unselective.

Table 1
The CF leakage from liposomes (values of the extent of the leakage, α, measured 100 s after the peptide addition are presented) induced by different peptides and their potency to induce the potassium current across a planar BLM (presented as the concentration which induced the conductance of 0.01 Ω^{-1} cm^{-2})

Peptide	α (%)	Concentration (ng/ml)
P1C: gramicidin-GSGRRRRSQS-COOH	85	30
P3C: gramicidin-GSGPKKKRKVG-CONH2	95	10
P2C: gramicidin-GSGPKKKRKVC-CONH2	90	15
P4C: gramicidin-(βA)-GSGPKKKRKVC-CONH2	65	60
P5C: Ac-gramicidin-(βA)-GSGPKKKRKVG-CONH2	50	1000
N1C: gramicidin-GSGEEEESQS-COOH	5	10
gA: gramicidin A	3	2

Peptide concentration in column 2 was 2 μg/ml. For other conditions, see Section 2.