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Relationship between gramicidin conformation dependent induction of phospholipid transbilayer movement and hexagonal H_{II} phase formation in erythrocyte membranes

Huibert Tournois [a], Ute Henseleit [b], Johannes De Gier [a], Ben De Kruijff [a,c] and Cees W.M. Haest [b]

[a] *Centre of Biomembranes and Lipid Enzymology and* [c] *Institute for Molecular Biology and Medical Biotechnology, University of Utrecht, Utrecht (The Netherlands) and* [b] *Department of Medical Physiology, RWTH Aachen, Aachen (F.R.G.)*

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Addition of gramicidin in sufficient concentration from dimethylsulfoxide or trifluoroethanol to isolated erythrocyte membranes induces hexagonal H_{II} phase formation for the phospholipids. In contrast, addition from ethanol does not change the overall bilayer organization despite a similar extent of peptide incorporation. The same solvent dependence is observed for the enhancement of transbilayer reorientation of lysophospholipids and unspecific leak formation in intact erythrocytes at lower gramicidin concentrations. These results indicate that the ($\beta^{6,3}$) conformation of the peptide is essential for all three membrane perturbing effects.

The topology of biosynthesis and the asymmetric distribution of phospholipids in biomembranes require a mechanism for the transbilayer movement of phospholipids (flip). Although this mechanism is largely unknown it is most likely a protein mediated process [1]. Since non-bilayer lipid structures can facilitate transbilayer transport of lipids in model systems [2,3], the possibility should be considered that protein-induced (transient) non-bilayer lipid structures are responsible for phospholipid flip in biomembranes. Experimental support for this hypothesis has been obtained in experiments with rat liver microsomes [4].

The hydrophobic peptide antibiotic gramicidin A is an attractive model to systematically investigate the interrelationships between peptide-induced transbilayer movements of lipids and membrane organization [5,6]. Gramicidin inserts spontaneously into the hydrophobic core of the membrane and forms, at low concentrations by N- to N-terminal dimerization of $\beta^{6,3}$ helices cation-selective channels [7,8]. The peptide enhances the transbilayer movement of lysophosphatidylcholines and palmitoylcarnitine in the erythrocyte membranes when the molar ratio of peptide to phospholipid exceeds 1:2000. This process is accompanied by an increase in permeation of intermediate sized solutes but not of high molecular weight dextrans [5]. Above a ratio of 1:80 part of

Abbreviations: CSA, chemical shift anisotropy; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, tris(hydroxymethyl)-aminomethane.

Correspondence: H. Tournois, Centre of Biomembranes and Lipid Enzymology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands.

the lipid of the erythrocyte membrane becomes organized in the hexagonal H_{II} phase [6] resulting in cell lysis. These effects are mechanistically related as formylation of the four tryptophans of the peptide or even a single Trp → Phe substitution, affects all of these properties likewise [5,6,9].

Recently it was demonstrated that the solvent history of gramicidin determines the conformation of the polypeptide upon incorporation in model membranes [10,11] and is crucial for its ability to induce H_{II} phase formation in dioleoylphosphatidylcholine (DOPC) model membranes [10]. It has been suggested that for the induction of the H_{II} phase the $\beta^{6.3}$ conformation, which prevails upon insertion from dimethylsulfoxide or trifluoroethanol, is a prerequisite [11]. Most likely the peptide inserts into the membrane out of an ethanolic solution as a relatively stable antiparallel dimer, which cannot induce H_{II} phase formation [10–12].

In this study we make use of this possibility to vary the conformation of gramicidin to investigate the relation between the gramicidin conformation and the induction of enhanced lipid transbilayer movement, unspecific leak formation and H_{II} phase formation in erythrocyte membranes.

Erythrocytes were isolated from fresh human blood anti-coagulated with citrate by centrifugation as described previously [5]. To protect gramicidin treated cells against colloid-osmotic lysis, incubations were carried out in media (90 mM KCl; 45 mM NaCl; 12.5 mM NaH_2PO_4/Na_2HPO_4 (pH 7.4)) supplemented with 40 mM Dextran 4 (M_r 4000–6000) (medium A).

Flip rates were measured at 37 °C by insertion of L-1-[1-^{14}C]palmitoyllysophosphatidylcholine (spec.act. 45 mCi/mmol) in the outer membrane layer of erythrocytes and measuring the gramicidin-dependent reorientation of lysophosphatidylcholine from the outer to the inner membrane layer by quantifying the increase in the fraction of lipid probe that becomes non-extractable by albumin [5,14]. Non-specific leak formation was measured by suspending erythrocytes in isotonic Hepes-buffered saline (at 37 °C, hematocrit 10%) containing 40 mM mannitol or sucrose to protect the cells against colloid-osmotic lysis as a result of specific channel-mediated salt uptake by the cells. Rates of influx of the solutes via non-specific leaks was monitored by the time-dependent increase of hemolysis [5]. The extent of binding of ^{14}C-labeled gramicidin (spec.act. 57 mCi/mmol) to erythrocytes was measured as described previously [5].

For NMR and freeze-fracture experiments white ghosts were prepared from human erythrocytes as described before [6]. Gramicidin (10 mM) in dimethylsulfoxide, ethanol or trifluoroethanol was added dropwise (flow rate approximately 40 μl/min) to 60 ml of a vigorously stirred dispersion of ghosts (20 μmol lipid phosphate) in buffer (150 mM NaCl, 10 mM Tris-HCl, 0.2 mM EDTA (pH 7.4)) at 20 °C. After 30 min of incubation at room temperature the ghosts were collected quantitatively by centrifugation (27000 × g, 20 min at 4 °C) and transferred to a NMR tube or taken for freeze-fracturing. In control experiments solvents without peptide were added.

Proton-noise decoupled ^{31}P-NMR spectra were recorded on a Bruker MSL-300 spectrometer at 121.5 MHz as described in [13] using an interpulse time of 1.0 second.

Freeze-fracture electron microscopy experiments were carried out as described in Ref. 6.

In agreement with earlier observations [6,15] dispersions of red cell membranes exhibit a broad anisotropic ^{31}P-NMR signal with a high-field peak and a low-field shoulder indicative for phospholipids organized in a lamellar, liquid crystalline phase. The effective ^{31}P chemical shift anisotropy (CSA) was 37 ± 1 ppm. This spectrum is not affected by the addition of amounts of solvent to be used to introduce gramicidin (Fig. 1A). In agreement with a previous study [6] addition of gramicidin (10 mol%) from dimethylsulfoxide leads to a dramatic change in lipid structure (Fig. 1B). The ^{31}P-NMR spectrum of these dispersions is a superposition of a bilayer component (CSA ~ 36 ± 1 ppm) and a signal with reversed asymmetry with a low-field peak at approximately + 3.4 ppm and a highly reduced CSA of 10 ± 1 ppm. This indicates that upon incorporation of gramicidin a fraction of the phospholipids is rearranged in a hexagonal H_{II} configuration. Addition from trifluoroethanol also results in comparable H_{II} phase formation (Fig. 1C). In contrast addition of 10 mol% gramicidin from ethanol does not influence the overall lipid organization (Fig. 1D). This is in

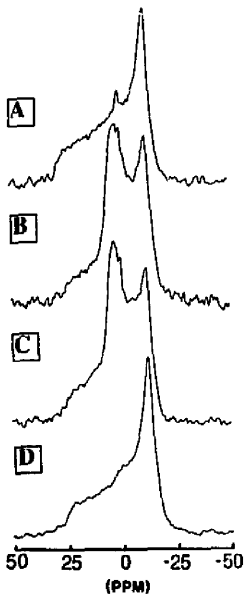

Fig. 1. 121.5 MHz ^{31}P-NMR spectra of aqueous dispersions of erythrocyte ghosts in the presence of 1% (v/v) ethanol (A) and ghosts after external addition of gramicidin (1:10 mol/mol phospholipid) from dimethylsulfoxide (B), trifluoroethanol (C) and ethanol (D) (final concentration of solvent < 1% (v/v). Spectra were recorded at 37°C and 3600–10000 free induction decays were accumulated.

agreement with the observation that gramicidin upon addition from ethanol does not induce H_{II} phase in DOPC [10] model membranes. Addition of larger quantities of gramicidin, even up to a 1:2 molar ratio with respect to the phospholipid content, did not lead to H_{II} phase induction. Also 24 h incubation at 37°C did not result in the H_{II} phase related ^{31}P-NMR signal (data not shown).

In freeze-fracture electron micrographs of erythrocyte ghost preparations treated with or without 1% (v/v) dimethylsulfoxide extended bilayers can be observed (data not shown). Addition of gramicidin 1/10 mol/mol phospholipid from dimethylsulfoxide or trifluoroethanol, in agreement with the NMR data, clearly show hexagonally organized tubes (data not shown). These structures are visible in both samples with glycerol as a cryoprotectant and in the rapid frozen samples in absence of cryoprotectant. On the other hand comparable structures could not be detected with either technique in samples of ghosts to which gramicidin was added from ethanol. In analogy with model membrane experiments [10] it can be suggested that the conformation of gramicidin inserted in the erythrocyte membrane from ethanol is unable to induce the H_{II} phase formation and that for H_{II} phase formation the $\beta^{6.3}$ conformation is essential.

The solvent history of gramicidin also has a marked influence on the transbilayer reorientation of lysophospholipids and palmitoylcarnitine. From Fig. 2 it is evident that the time-dependent transbilayer reorientation of lysophosphatidylcholine is highly accelerated by 5 μmol/l gramicidin, when added from dimethylsulfoxide, but not when added from ethanol. The first enhancement of flip by gramicidin added from ethanol is observed above 5 μmol/l (inset of Fig. 2), which is a 10-fold higher concentration than that required when the peptide is added from dimethylsulfoxide [5]. Flip-

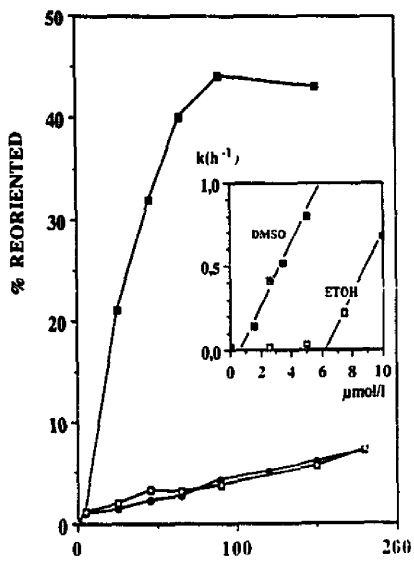

Fig. 2. Acceleration of the time-dependent transbilayer reorientation of [^{14}C]palmitoyllysophosphatidylcholine by gramicidin (5 μmol/l ≈ 1:200 mol/mol phospholipid). Inset: Concentration-dependent increase of flip-rate constants (at 37°C) for palmitoyllysophosphatidylcholine by gramicidin. The data points represent mean values of 2–5 experiments. Gramicidin is added from either dimethylsulfoxide (■) or ethanol (□). Control without gramicidin (●).

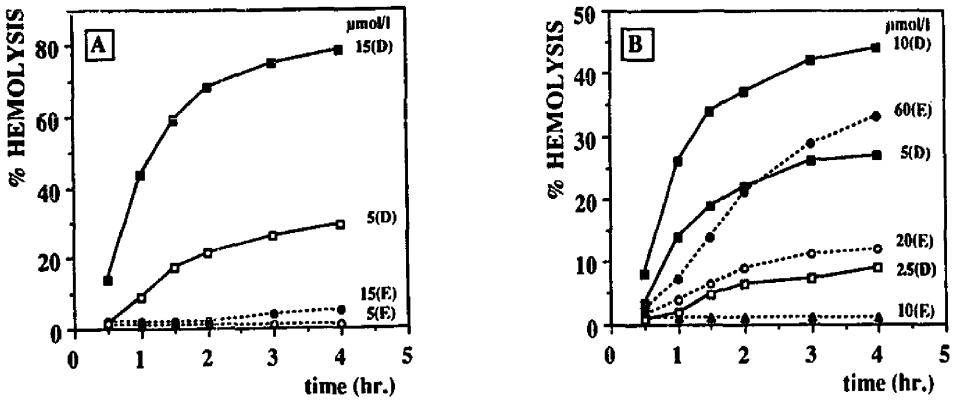

Fig. 3. Protection of erythrocytes against gramicidin-induced lysis by sucrose (A) and mannitol (B). The time-course of hemolysis (at 37°C) was followed after addition of gramicidin from dimethylsulfoxide (———) or ethanol (— — —) at the concentrations indicated in the figure. E, ethanol; D, dimethylsulfoxide.

site formation by gramicidin added from dimethylsulfoxide has been shown to be paralleled by an increase in non-specific permeability of the membrane for non-electrolytes (mannitol, sucrose) and ions (choline, oxalate) [5]. Addition of gramicidin from dimethylsulfoxide induces a colloid-osmotic hemolysis of erythrocytes due to the formation of ion-channels in the membrane [5]. Comparable hemolysis was induced by gramicidin added from ethanol (data not shown). At concentrations up to 0.5 μmol/l, the gramicidin-induced hemolysis could be completely suppressed by the addition of sucrose or mannitol to the medium (data not shown). However above this concentration, addition of gramicidin from dimethylsulfoxide but not from ethanol produces non-specific membrane-leaks for sucrose as can be inferred from the resulting hemolysis (Fig. 3A). Upon addition of gramicidin from ethanol, 10-fold higher concentrations are required to obtain comparable leaks for mannitol as obtained for gramicidin added from dimethylsulfoxide (Fig. 3B).

That the differences shown are not the result of differences in peptide incorporation into the membrane can be inferred from the binding experiments using ^{14}C-labeled gramicidin. After addition of the peptide at ratios of 1:200, 1:100 and 1:50 mol/mol phospholipid from ethanol or dimethylsulfoxide, on average, respectively, 68% and 72% of the radioactivity could be co-pelleted with the membranes by centrifugation.

It has been suggested [6] that H_{II} phase formation and flip-enhancement are mechanistically related events and that gramicidin aggregates of specific but yet unknown structure, which are considered to be precursors gramicidin-induced H_{II} phase formation [12] are involved in both processes. In the present study we show that the $\beta^{6.3}$ conformation of the gramicidin is a prerequisite for its lipid-structure modulating and flip-enhancing abilities. This can be explained by the differences in dynamical shape between the gramicidin molecules in the $\beta^{6.3}$ and an anti-parallel β conformation. Of these, only the $\beta^{6.3}$ helix, due to the location of all four tryptophans near the C-terminus of the molecule has a pronounced cone shape and may have the ability to form membrane perturbing aggregates [12].

An important biological implication of the present data might be that the mechanism of action of a flippase could involve a change in polypeptide conformation triggering the flip-promoting activity.

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