Effect of lipids with different spontaneous curvature on the channel activity of colicin E1: evidence in favor of a toroidal pore

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Abstract The channel activity of colicin E1 was studied in planar lipid bilayers and liposomes. Colicin E1 pore-forming activity was found to depend on the curvature of the lipid bilayer, as judged by the effect on channel activity of curvature-modulating agents. In particular, the colicin-induced *trans*-membrane current was augmented by lysophosphatidylcholine and reduced by oleic acid, agents promoting positive and negative membrane curvature, respectively. The data obtained imply direct involvement of lipids in the formation of colicin E1-induced pore walls. It is inferred that the toroidal pore model previously validated for small antimicrobial peptides is applicable to colicin E1, a large protein that contains ten α-helices in its pore-forming domain.

Keywords: Bilayer lipid membrane; Spontaneous curvature; Ion channel; Toroidal pore; Colicin E1; *Trans*-membrane current; Dye leakage; Tryptophan fluorescence

1. Introduction

Colicin E1 belongs to a group of water-soluble ''membrane-active'' proteins that can interact with lipid membranes through conformational changes [1–3]. These interactions result in the formation of ion-conducting pores in lipid bilayers [4–6]. It has been shown that pore formation by the closely related colicins Ia and A is accompanied by translocation of a substantial part of the colicin polypeptide across the membrane [7–11]. Moreover, hydrophilic proteins inserted in the translocated segment of the pore-forming colicin are themselves translocated and are functional on the opposite side of the bilayer [12]. However, the structure of the pore and the mechanism of translocation remain obscure.

Progress in the research of the action of antimicrobial peptides on membranes has led to the formulation of the model of a toroidal lipid pore [13], involving direct participation of lipids in the formation of the pore [14–25]. An essential feature of this model is that a wall of the pore consists not only of peptides but also of lipid headgroups, which line the pore from one side of the membrane surface to the other, thereby forming a structure of high positive curvature.

The toroidal pore mechanism has been considered for a series of small antimicrobial peptides, e.g., magainin [16], melittin [23], pleurocidin [26], the phytotoxic lipopeptide syringomycin E [27], the human cathelicidin antimicrobial peptide LL-37 [28], and the hagfish cathelicidin antimicrobial peptide [29]. The major evidence in favor of toroidal pore formation by the peptides derives from the sensitivity of their pore-forming activity to the curvature of the target lipid bilayer membranes. Lipids with positive spontaneous curvature (SC) stimulate, while lipids having negative SC inhibit the formation of pores by magainin and syringomycin [16,27]. The value of the SC, determined by the ratio of the cross-sectional areas of headgroup to acyl chain moieties [30,31], characterizes the ability of lipids to form non-bilayer structures.

The formation of a structure closely related to a toroidal pore, with highly bent transient lipidic connections between fusing membranes (the so-called fusion stalks) is implicated in the process of membrane fusion [32–35]. In particular, it has been shown that the agents having an ability to modify the SC of a planar lipid bilayer, lysophosphatidylcholine and oleic acid (OA), inducers of positive and negative SC, respectively, inhibit and stimulate the fusion of cells with a target planar membrane [33]. The electrical breakdown of planar bilayer lipid membrane (BLM) is also thought to occur through expansion of positively curved toroidal-type lipidic pores [36].

The toroidal pore concept has also been proposed to be involved in the mechanism of action of large toxins belonging to a group of actinoporins, e.g., equinatoxin II [37] and sticholysins [38,39], although the influence of membrane curvature has shown to be opposite for sticholysin [38] compared to magainin [16]. The authors suggest that the presence of a minor amount of cardiolipin, a strong inducer of negative SC,

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Abbreviations: BLM, bilayer lipid membrane; DPhPC, diphytanoylphosphatidylcholine; DPhPG, diphytanoylphosphatidylglycerol; DPhPS, diphytanoylphosphatidylserine; DOPC, dioleoylphosphatidylcholine; DOPG, dioleoylphosphatidylglycerol; DOPE, dioleoylphosphatidylethanolamine; M-LPC, myristoyl-lysophosphatidylcholine; O-LPC, oleoyl-lysophosphatidylcholine; OA, oleic acid; 9,10-BrPC, 9,10-dibromo-palmitoylphosphatidylcholine; CF, carboxyfluorescein; SC, spontaneous curvature

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augments the efficiency of toxin pore formation because of the presence of a negative curvature region in the plane of the membrane around the toroidal pore. Recently, evidence has been reported pointing to the formation of toroidal-shaped pores by the pro-apoptotic proteins Bax [40,41] and tBid [42], though the data on the relationship between the bilayer SC and the membrane-permeabilizing potency of these proteins are rather diverse [41–44].

In the present study, we used agents that modulate the membrane SC in order to test their effect on colicin E1 channel function. It was shown that lysophosphatidylcholine promoted the colicin channel activity, whereas OA reduced it. In addition, experiments with membranes of different lipid compositions revealed a correlation between the membrane permeabilizing potency of colicin and the bending propensity of a bilayer. Thus, the data obtained here point to the relevance of the toroidal pore mechanism to the process of colicin E1 channel formation.

2. Materials and methods

The 178-residue C-terminal colicin E1 channel polypeptide, P178, was prepared by thermolysin proteolysis of intact colicin E1 [45].

Planar BLMs were formed from a 2% solution of diphytanoylphosphatidylcholine/diphytanoylphosphatidylglycerol (DPhPC/DPhPG) (70/30 mol%) in squalene by the brush technique [46] on a 0.55-mm diameter hole in a Teflon partition separating two compartments of a cell containing aqueous solutions of 120 mM KCl and 10 mM β-alanine, pH 4.0. 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 60 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. C-terminal colicin peptide, P178 [45], was added to the *cis*-side of the membrane, and myristoyl-lysophosphatidylcholine (M-LPC) and water-soluble OA (a complex of OA and methyl-β-cyclodextrin, Sigma) were added to both sides of the BLM.

Dye-loaded liposomes were prepared by evaporation under a stream of nitrogen of a 2% solution of a mixture of lipids (Avanti Polar Lipids, AL) in chloroform followed by hydration with a buffer solution containing 250 mM Tris and 100 mM carboxyfluorescein (CF). The mixture was vortexed, passed through a cycle of freezing and thawing, and extruded through 0.1-μm pore size Nucleopore polycarbonate membranes using an Avanti Mini-Extruder. The unbound CF was then removed by passage through a Sephadex G-50 coarse column with a buffer solution containing 10 mM β-alanine and 0.12 M KCl, pH 4.0.

Binding of P178 to membranes was monitored using liposomes containing brominated lipids known to quench tryptophan fluorescence [2,47,48]. Liposomes with high 9,10-dibromo-palmitoylphosphatidylcholine (9,10-BrPC) content were made from a 2 % solution of 9,10-BrPC/DPhPG (70/30 mol%) in chloroform. The buffer solution used for preparation of the brominated liposomes contained 10 mM β-alanine and 0.12 M KCl, pH 4.0. The same buffer was used in experiments on tryptophan fluorescence quenching by the brominated liposomes.

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: $\alpha\ (\%) = 100 \cdot (F_f - F_0)/(F_{100} - F_0)$, where F_0 and F_f represent the initial and the final (steady-state) levels of fluorescence before and after the protein addition, respectively, 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 β-alanine buffer (10 mM β-alanine and 0.12 M KCl, pH 4.0) was used for the fluorescence studies.

Colicin (P178) was added from a stock water solution to the buffer solution in the spectrophotometric cuvette. M-LPC and OA were added to the buffer solution containing dye-loaded (measurements of CF leakage) or brominated (measurements of tryptophan fluorescence quenching) liposomes in the cuvette. Tryptophan fluorescence excited at 280 nm was measured at 327 nm (band-pass of both beams, 5 nm). All experiments were performed at room temperature.

3. Results

3.1. Colicin E1 currents across planar lipid bilayers

The effects of M-LPC and OA on the macroscopic current across BLM mediated by the channel-forming domain of colicin E1 (P178) are demonstrated in Fig. 1, panels A and B, respectively. It is seen that M-LPC induced the increase in the colicin E1-mediated current, while OA had the opposite effect.

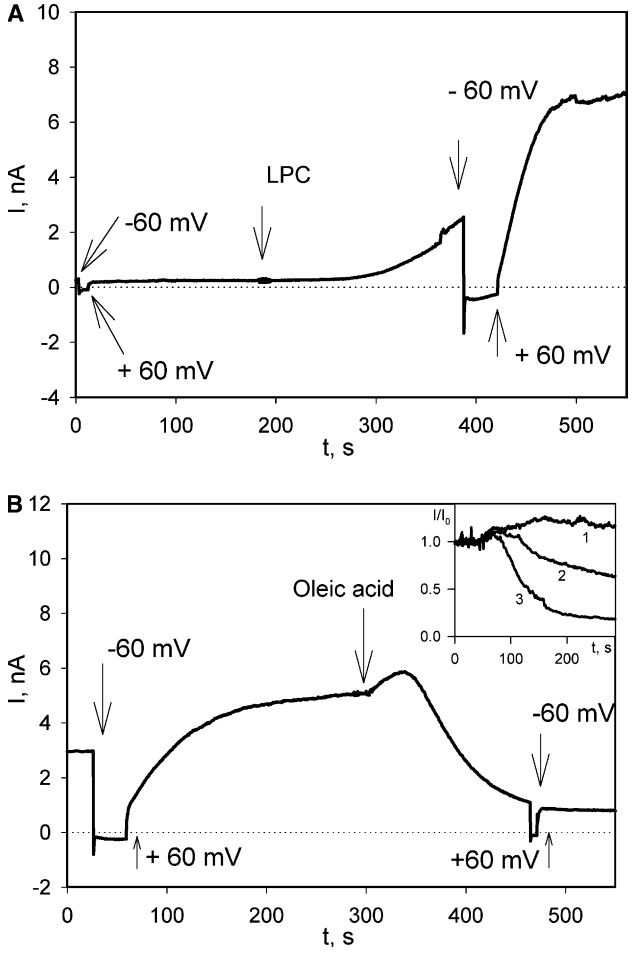

Fig. 1. Effect of 10 μM M-LPC (panel A) or 10 μM OA (panel B) on the colicin E1-induced current across a planar lipid bilayer. The membrane was formed from a squalene solution of DPhPC/DPhPG (70/30%). The buffer solution was 10 mM β-alanine and 120 mM KCl, pH 4.0. Initial voltage was 60 mV. Inset to panel B. Dependence of the colicin E1-induced current across a planar lipid bilayer on OA concentration. The concentration of OA was 10 μM (curve 2) and 30 μM (curve 3). Curve 1, control in the presence of methyl-β-cyclodextrin at the same concentration (30 μM) as in curve 3. The membrane was formed from a squalene solution of DPhPC/DPhPG (70/30%). Initial voltage, 60 mV. Current is plotted relative to initial conditions.

Importantly, colicin E1 current retained the voltage-dependence after the addition of both M-LPC and OA (voltage changes are marked by arrows). It should be noted that the decrease in the current induced by OA was often preceded by a transient activation (Fig. 1B). OA was added to the bathing solution in the form of a complex with cyclodextrin called "water-soluble OA" (complex of OA and methyl-β-cyclodextrin) to provide better delivery and insertion into the membrane of OA. The control experiments showed that cyclodextrin itself did not increase the conductivity of the membrane and produced no effect on the colicin-induced current (Fig. 1B, inset, curve 1). The inset to Fig. 1B illustrates the time courses of the current suppression by increased concentrations of OA (curves 2 and 3). The effective concentrations (3–30 μM) were close to those stimulating membrane fusion mediated by influenza hemagglutinin (1–10 μM) [33].

Single channels of P178 recorded in DPhPC bilayers at 1 M KCl exhibit the voltage dependence similar to that characteristic of the macroscopic current [49]. Fig. 2 illustrates typical recordings of colicin E1 channels in the control (A) and in the presence of M-LPC (B) and oleic acid (C). The single-channel analysis (not shown) revealed that the channel amplitudes remained unaltered after the addition of M-LPC or OA, whereas the number of open channels differed considerably in the presence of these agents, increased and decreased, respectively, in the presence of M-LPC and OA.

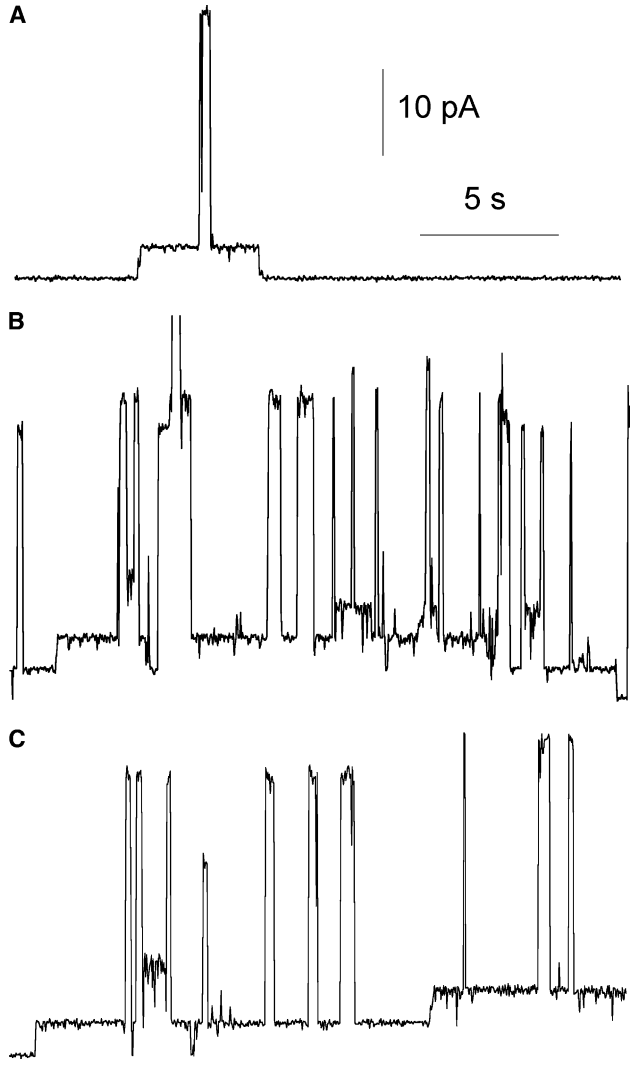

Fig. 2. Single-channel traces of colicin E1 (panel A), in the presence of 20 μM M-LPC (panel B) and 30 μM OA (panel C). The membrane was formed from a decane solution of DPhPC. The buffer solution was 10 mM β-alanine and 1 M KCl, pH 4.0. The voltage of 80 mV was applied to BLM.

3.2. CF leakage from liposomes

The dimensions of pores formed in BLM by antimicrobial peptides and toxins are so large that they allow the passage of bulky molecules as CF and calcein [15,16,29,37,38,50–54]. In this work, colicin E1-induced leakage of CF was measured from liposomes with different membrane curvatures. The extent of the CF leakage constituted about 17% and depended slightly on the concentration of P178 within a range 5–40 nM. In contrast, the rate of the CF leakage, as judged by the half-time ($t_{1/2}$) of the time course, rose markedly as the P178 concentration was increased from 10 nM ($t_{1/2} = 180$ s) to 40 nM ($t_{1/2} = 10$ s), in agreement with earlier data obtained with the intact colicin E1 protein [55].

Dye-release experiments showed that the rate of the colicin-induced CF leakage substantially increased in the presence of M-LPC (Fig. 3), which is consistent with the effect of this SC-modulating agent on the colicin-induced macroscopic current (Fig. 1). The half-time for the CF leakage decreased markedly over a concentration range of 1–10 μM M-LPC at a P178 concentration of 10 nM (Fig. 3, inset). The effective concentrations were close to those stimulating membrane fusion mediated by influenza hemagglutinin (3–30 μM) [33].

To further test the influence of membrane SC on the colicin channel-forming activity, experiments were performed with liposomes, formed from lipids with different SC. Fig. 4A shows the time courses of the colicin-induced CF leakage of liposomes containing oleoyl-lysophosphatidylcholine (O-LPC) or dioleoylphosphatidylethanolamine (DOPE) as compared to that of the control liposomes formed from dioleoylphosphatidylcholine/dioleoylphosphatidylglycerol (DOPC/

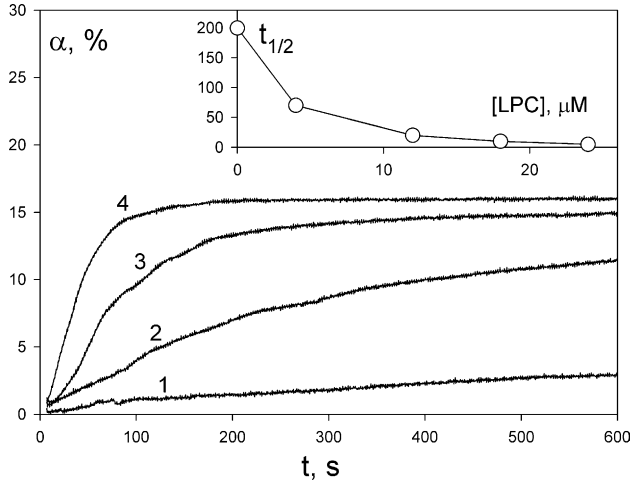

Fig. 3. The effect of M-LPC on the CF leakage (α denotes the extent of the CF leakage). The concentration of the DPhPC/DPhPG (70/30%) liposomes was 10 μM. The concentration of P178 was 5 nM. Curve 1, control; curves 2–4, M-LPC added at concentrations of 2, 4 and 12 μM, respectively. Inset: Dependence of $t_{1/2}$ of CF leakage on the concentration of M-LPC (P178 concentration here was 10 nM).

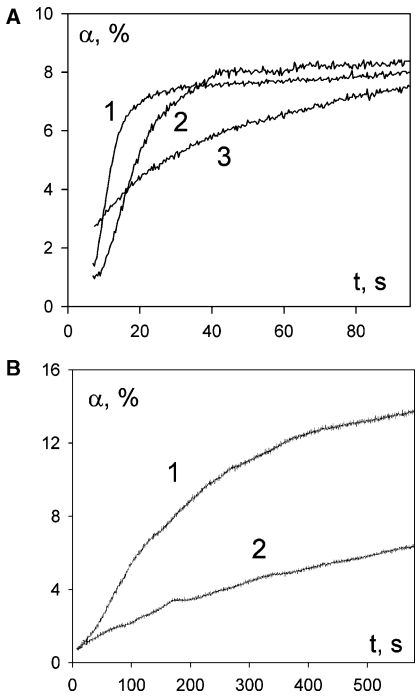

Fig. 4. CF leakage from liposomes with different lipid compositions. The concentration of P178 was 10 nM, other conditions as in Fig. 2. (A) O-LPC/DOPC/DOPG (2:5:3), curve 1; DOPC/DOPG (7:3), curve 2; DOPE/DOPG (7:3), curve 3. (B) DPhPC/DPhPG (7:3), curve 1; DPhPC/DPhPS (7:3), curve 2.

DOPG) (70%/30%, w/w). It is known that lysophosphatidylcholine having a relatively large polar head group and only one acyl chain is characterized by a high value of positive SC [56,57], while DOPE with a small polar head group and two acyl chains has a high negative SC [57,58]. It is seen that the colicin-induced CF leakage was accelerated in the presence of O-LPC (Fig. 4A, curve 1) and decelerated in the presence of DOPE (Fig. 4A, curve 3) relative to the control (Fig. 4A, curve 2). It should be mentioned that apart from the slow phase, the time course of the colicin-induced CF leakage of DOPE/DOPG liposomes contained a fast response that could not be resolved (Fig. 4A, curve 3). Consistent with the difference in the headgroup surface areas of hydrated phosphatidylglycerol and phosphatidylserine [59], Fig. 4B shows that liposomes formed from DPhPC/diphytanoylphosphatidylserine (DPhPS) (7:3) exhibited a slower colicin-induced CF leakage than those formed from DPhPC/DPhPG (7:3). We note that the surface areas were compared at higher pH than was used in the present study and have assumed that the difference also applies to the conditions used here. From the comparison of Fig. 4B, curve 1 with Fig. 4A, curve 2, it is evident that the permeabilizing potency of colicin E1 is reduced in DPhPC/DPhPG compared to DOPC/DOPG liposomes, in accord with the fact that the cross-sectional area of the diphytanoyl tail is larger than that of the dioleoyl tail [60,61]. This conclusion is supported by corresponding measurements of the colicin E1-mediated current with DPhPC/DPhPG and DOPC/DOPG membranes (data not shown). In addition, experiments were carried out with cardiolipin, which has more massive fatty acid body. Cardiolipin did not affect the rate of CF release (not shown), although it is known to induce negative SC. The reason might be that the size of cardiolipin is too large to incorporate in the toroidal pore.

3.3. Colicin E1 binding to liposomes

It is known that the binding of colicin to membranes is governed by electrostatics [62] and thus should not be sensitive to membrane SC. We probed the effect of SC-modulating agents on the colicin-membrane binding in experiments that utilized tryptophan fluorescence quenching by brominated phospholipids. Fig. 5 shows a typical time course of the P178 fluorescence after the addition of liposomes containing 9,10-BrPC. From the comparison of the corresponding traces of the fluorescence recorded in the presence of the SC-modulating agents, it can be concluded that neither M-LPC nor oleic acid affected the binding of P178 to the lipid bilayer (Fig. 5, inset). The quenching of colicin tryptophan fluorescence by brominated phospholipids proceeded much faster than the colicin-induced CF release from liposomes (compare with Fig. 3). Upon addition of liposomes to the protein solution, association of the channel-forming domain of colicin E1 with the vesicles is, without stopped-flow-type measurements, apparently instantaneous and thus too fast to be detected by our present experimental system.

4. Discussion

The results of this study have demonstrated the correlation between the colicin channel-forming activity and membrane curvature, thereby supporting the hypothesis that channel formation by colicin E1 occurs via a mechanism involving toroidal lipidic pore. This mechanism implies direct involvement of lipid headgroups in the formation of the channel wall. It can be proposed that, in contrast to antimicrobial peptides, the formation of the colicin E1 channel does not require oligomerization of the protein, since it has 8 hydrophilic-amphypathic and 2 hydrophobic α-helices in its bound state which should be sufficient for the formation of a toroidal pore.

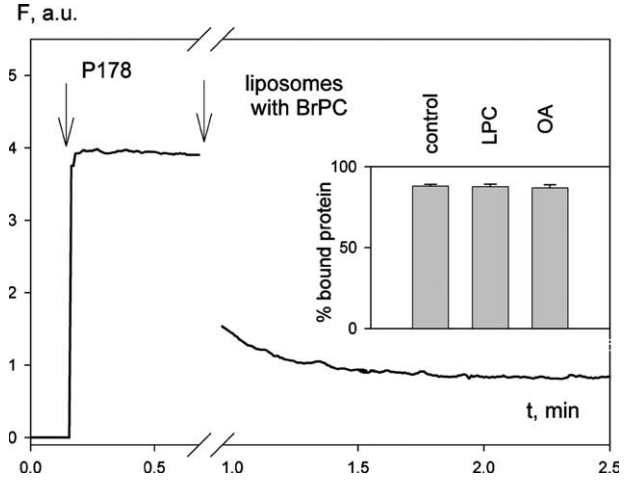

Fig. 5. Binding of colicin E1 to liposomes. Time course of the tryptophan fluorescence quenching by BrPC-containing liposomes. Concentrations of P178, 100 nM, and brominated liposomes, 150 μM. Inset: The extent of quenching of the P178 fluorescence by the brominated liposomes without additions, in the presence of 15 μM M-LPC and 20 μM OA, respectively.

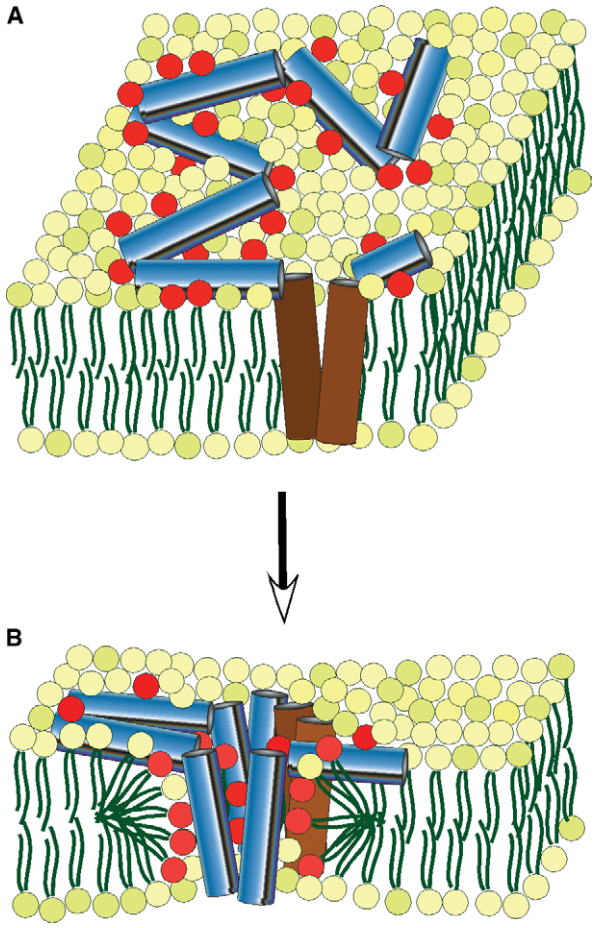

Fig. 6. A scheme of formation of toroidal pore in lipid bilayer induced by colicin E1. (A) Closed state of pore after colicin binding to membrane or after pore closing in the presence of a *trans*-positive potential. (B) Toroidal configuration of the open pore state. Colicin α-helices I–VII and X are shown in blue, a hairpin of VIII–IX-helices is shown in brown. Headgroups of neutral lipids are shown in yellow, while those of anionic lipids are shown in red. Because the anionic lipid involved in the toroidal channel structure is proposed to be paired with basic residues in the *trans*-membrane segments of colicin, it would not contribute to a net negative charge that would cause a preference of the channel for cations.

In the case of the peptide magainin, the toroidal pore consists of 4–7 α-helices, as estimated from neutron-scattering data [14]. Fig. 6 illustrates a scheme of the toroidal pore formed by colicin E1. The walls of the pore are made not only of colicin α-helices (two hydrophobic and four additional helices), but also of phospholipid molecules intercalated between them. These lipid molecules experience elastic strain if placed in highly curved structures. Hence, pore formation should be facilitated by including lipids with high positive values of SC, e.g., lysophospholipids.

It is noteworthy that the data on the stimulation of the colicin E1 channel-forming activity by lysophosphatidylcholine, the agent inducing positive SC, apparently explain the stimulating effect of *N*-bromosuccinimide (NBS) treatment of BLM on the colicin E1-mediated current observed in [49]. The interaction of NBS with unsaturated phospholipids leads to the formation of bromohydrins [63] that are suggested to undergo subsequent hydrolysis of an ester bond, thus producing lysophospholipids similarly to chlorohydrins [64].

According to the toroidal pore structure, the phospholipids in such a pore would exhibit negative curvature in a dimension parallel to the bilayer plane but positive curvature along the bilayer normal [17]. Thus, the toroidal pore formation could be stimulated by lipids characterized by negative SC, as it was observed with sticholysin [38]. This may explain the transient stimulation of colicin-induced current after the addition of OA (Fig. 1).

As mentioned in Section 1, alteration of membrane curvature results in different functional consequences, e.g., modulation of membrane fusion [33], and regulation of the activity of mechanosensitive channels [65,66], sensing of membrane-bending events by a conserved protein BAR domain that is crucial for processes of membrane remodeling in growing cells [67], regulation of the assembly/disassembly cycle of the COPI coat on Golgi membranes [68]. A number of studies have shown that the agents modifying membrane SC can change the characteristics of peptidic ion channels. In particular, the addition of lysophospholipids inducing positive membrane curvature causes an increase in both the number and the lifetime of open gramicidin channels [69]. Recently, Bruno et al. [70] have demonstrated that the addition of OA, known to produce the opposite effect on membrane curvature, also provokes an increase in gramicidin channel lifetime. Based on these results, the authors came to the conclusion that the mechanical properties of the bilayer, not simply curvature, determine the gramicidin channel characteristics that are associated with hydrophobic mismatch between the channel length and the bilayer thickness [71].

Keller et al. [72] and Bezrukov et al. [73] observed a correlation between membrane SC and the relative probabilities of different conductance states of alamethicin channels. In particular, states of higher conductance were more probable in DOPE, a lipid with high negative curvature, than in DOPC, a lipid with low curvature. These results were explained in terms of the "barrel-stave" model by linking a degree of peptide oligomerization to a membrane-induced line tension [72,74]. According to our measurements, the dependence of colicin E1 single-channel activity on the membrane SC differed substantially from that of alamethicin channels. Modulation of SC led to alteration of the total number of open channels, but did not change the distribution of probabilities of low- and high-conductance states.

The dependence of ion selectivity of colicin channels on the presence of negatively charged lipids in the bilayer [75] can also be interpreted in terms of direct participation of lipid molecules in the colicin pore walls or, alternatively as an effect of membrane surface potential. The idea of involvement of lipid molecules in the colicin pore structure has been proposed by Kienker et al. [11]. Actually, the toroidal pore model discussed here readily explains the ability of pore-forming colicins to translocate their long segments across a membrane [8], by predicting that the access to the *trans*-membrane helices of the pore-forming colicins will be larger than in a pure protein pore model.

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