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Induction of Lipid Flip-Flop by Colicin E1 – a Hallmark of Proteolipidic Pore Formation in Liposome Membranes

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Abstract—The addition of the channel-forming domain of colicin E1 to liposomes elicited the transmembrane diffusion (flip-flop) of lipids concomitant to the release of the fluorescent dye from liposomes. Good correlation was found between kinetic and concentration dependences of the two processes. Both the liposome leakage and the lipid flip-flop were stimulated upon alkalinization of the buffer solution after colicin binding at acidic pH. These results in combination with the analysis of the data on colicin binding to liposomes provide evidence in favor of the validity of the toroidal (proteolipidic) pore model as the mechanism of colicin channel formation.

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Key words: colicin, ion channel, liposome leakage, proteolipidic pore, flip-flop, fluorescence quenching

Accumulation of data on effects of antimicrobial peptides has resulted in development of a number of concepts concerning mechanisms of their interactions with biological membranes [1]. Works investigating colicin E1 bactericidal protein exemplify this direction. In particular, results of investigations of its pore-forming activity showed that this protein forms ion channels in lipid membranes following the toroidal pore mechanism [2-4] earlier proposed for a number of membrane-active peptides such as magainin and mellitin [5-7]. This mechanism is based on direct involvement of lipid head groups along with protein helices in formation of the ion channel walls. The applicability of the toroidal pore model to colicin E1 was confirmed in a number of works [8, 9]. Data showing the validity of such a model for the colicin E1 channel include, first of all, the dependence of its pore-forming activity on the lipid membrane curvature [2, 10] and significant stimulation of lipid flip-flop (transmembrane diffusion) by this protein [3].

Abbreviations: BLM, bilayer lipid membrane; Br-PC, 9,10-dibromopalmitoylphosphatidylcholine; CF, 5(6)-carboxyfluorescein; DPhPC, diphytanoyl phosphatidylcholine; DPhPG, diphytanoyl phosphatidylglycerol; P178, channel-forming domain of colicin E1; PyPC, 1-lauroyl-2-(1-pyrenebutyroyl)-sn-glycero-3-phosphatidylcholine.

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It has been shown [2, 11] that addition of colicin E1 to a suspension of liposomes loaded with a fluorescent dye results in the release of the dye into the aqueous solution, which is indicative of pore formation in the liposomal membranes because no destruction of vesicles is observed [12]. In this case the liposome content release is judged by the increase in fluorescence of the dye present inside liposomes in high concentration sufficient for fluorescence quenching [13]. The colicin pore-forming activity in liposomes, measured in this way, essentially depends on the membrane lipid composition: incorporation into vesicles of lipids characterized by high spontaneous curvature results in significant stimulation of colicin-induced dye release from the liposomes [2]. This important result favoring the toroidal model of the colicin channel is supported by the observation of colicin-induced flip-flop of lipids in liposomes [3] registered on liposomes containing in the external monolayer the pyrene-labeled analog of phosphatidylcholine—1-lauroyl-2-(1-pyrenebutyroyl)-sn-glycero-3-phosphatidylcholine (PyPC) [14]. Addition of P178 (the channel-forming domain of colicin E1, 178 amino acid residues) to liposomes at pH 4.0 resulted in redistribution of fluorescent lipid analog between vesicle monolayers, which was expressed in the decreased ratio of intensities (I_E/I_M) of fluorescence spectrum peaks belonging, respectively, to pyrene excimers (I_E) and monomers (I_M). For further substantiation of the colicin

channel toroidal model, we studied the relationship between different manifestations of colicin E1 membrane activity, namely between pore-forming activity of this protein and stimulation of flip-flop of lipids.

MATERIALS AND METHODS

Colicin E1 C-domain (P178) obtained by proteolysis of whole colicin E1 by thermolysin [15] was provided by S. D. Zakharov (Purdue University, USA). All lipids used in this work were obtained from Avanti Polar Lipids (USA), and 5(6)-carboxyfluorescein (CF) was from Molecular Probes (USA).

PyPC was synthesized according to an earlier described technique [14]. One equivalent of 1-lauroyl-2-hydroxy-sn-glycero-3-phosphatidylcholine (Avanti) and four equivalents of 1-pyrenebutanoic acid (Aldrich, USA) were dried for 3 h under high vacuum, dissolved in freshly-distilled chloroform, and two equivalents of 4-(dimethylamino)-pyridine (Merck, Germany) and five equivalents of N,N′-dicyclohexylcarbodiimide (Sigma, USA), also in the form of solution in chloroform, were added to the resulting solution. The mixture was incubated for 8 h in the dark at 25°C under continuous stirring under an atmosphere of nitrogen, and then the dicyclohexylurea was filtered off. The filtrate was separated by preparative TLC on silica gel 60 F_{254} plates (Merck) by elution with chloroform–methanol–water mixture (50 : 25 : 4 v/v). The plate was developed under ultraviolet light. The product-containing region (R_f ~ 0.45) was scraped off, and the product was extracted from silica gel by elution mixture used earlier for chromatography. The product was additionally purified by RP-HPLC on a 250 × 8-mm C4 column (Vydac, USA) using isocratic elution by a mixture of 30% MeOH with 70% acetonitrile. Absorption was measured at 344 nm. The product was characterized by RP-HPLC on the same column using isocratic elution by the same mixture, by analytical TLC on Kieselgel 60 F_{254} (Fluka, Germany) plates with mixture applied earlier for preparative TLC (chloroform–methanol–water (50 : 25 : 4 v/v) as eluent), as well as by MALDI TOF/TOF mass spectrometry with α-cyan-4-hydroxycinnamic acid as matrix. The purified product was eluted as a single RP-HPLC peak (retention volume 26 ml) and a single spot on TLC (R_f = 0.45). The monoisotopic ratio obtained by mass spectrometry was m/z = 709.7 (m/z_{theor} = 710). The product yield was 50% of theoretical level on the basis of the lysophospholipid amount taken for reaction.

Liposomes loaded with CF were prepared from lipid solution in chloroform. After evaporation of chloroform in a nitrogen flow and lipid hydration by solution containing 100 mM CF the mixture was shaken, and after freezing–thawing cycle it was pressed through a polycarbonate membrane with 0.1 μm pores (Nucleopore) using a microextruder (Avanti). Unbound dye was removed by passing the liposomes through a column of Sephadex G-50. The buffer solution contained 10 mM β-alanine, 10 mM MES, 10 mM Tris, and 0.12 M KCl, pH 4.0.

Dye release from liposomes (α) was measured by fluorescence increase (removal of concentration quenching) calculated using the following formula:

$$\alpha\ (\%) = 100 \cdot (F_f - F_0)/(F_{100} - F_0),$$

where F_0 and F_f are initial and final fluorescence levels, respectively, before and after addition of the protein, while F_{100} is the fluorescence level after complete destruction of the liposomes by addition of detergent (lauryldimethylamine-N-oxide (LDAO) to concentration of 2.4% in experiments at pH 4.0 or Triton X-100 to 0.1% concentration in experiments at pH 6.0).

To study flip-flop of lipids, we used the system with pyrene-labeled phosphatidylcholine (PyPC) analog described in [14]. As seen in Fig. 1, the fluorescence spectrum of PyPC incorporated in the outer monolayer of lipid vesicles is characterized by several bands (dotted line), among which a number of narrow short-wavelength peaks correspond to PyPC monomer (let us designate intensity of one of them as I_M), and a broad long-wavelength band (with intensity I_E in the maximum) corresponds to PyPC excimers. The process of lipid flip-flop resulting in PyPC redistribution between liposome monolayers is accompanied by decrease in intensity of the peak corresponding to excimers and increase in the peaks corresponding to PyPC monomers (Fig. 1, continuous line). To incorporate PyPC exclusively into the external liposome monolayer, PyPC dissolved in methanol was

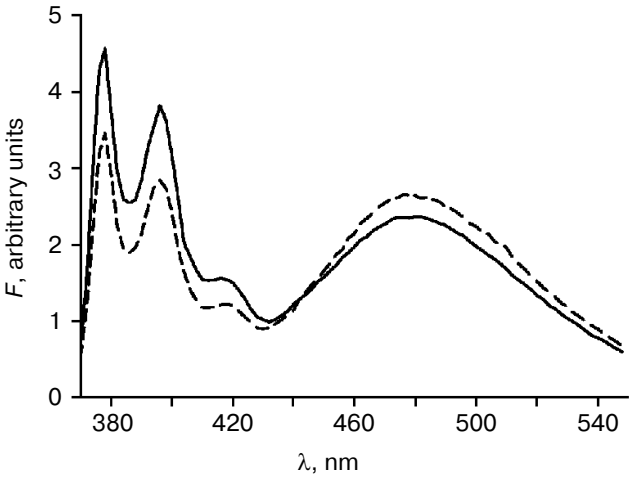

Fig. 1. Fluorescence spectrum of PyPC-containing liposomes before (dotted line) and after (continuous line) addition of P178. P178 facilitates flip-flop of PyPC, which lowers intensity of the peak corresponding to PyPC excimers and enhances peaks corresponding to PyPC monomers.

added to buffer solution containing the liposomes. To determine the I_E/I_M ratio, fluorescence levels at 480 (I_E) and 396 nm (I_M) were measured. The excitation wavelength was 344 nm. The flip-flop kinetics was judged by the change in the I_E/I_M ratio in time. For quantitative estimation of the kinetics, the $t_{1/2}$ (the time necessary for flip-flop to achieve 50% of maximally possible under these conditions) as well as τ, the exponent index in description of kinetic curves by exponential function (the time required for the measured signal to decrease e-times) were used.

The binding of P178 to the membrane was measured using liposomes containing brominated lipid that is known to quench tryptophan fluorescence. Liposomes containing 9,10-dibromopalmitoylphosphatidilcholine (Br-PC) were prepared from 2% solution of Br-PC/DPhPG (diphytanoyl phosphatidylglycerol) (70/30 mol%) in chloroform.

Fluorescence was measured on a Hitachi F-4000 or Fluorat-02 Panorama fluorimeter (Lumex, Russia) (wavelengths for CF excitation and emission are 490 and 520 nm, respectively).

RESULTS

We compared kinetic and concentration dependences of two processes indicating colicin E1 interaction with membrane lipids: dye release from liposomes and transmembrane lipid diffusion.

Figure 2 shows kinetic curves of the release from liposomes of the fluorescent dye CF (a) and of transmembrane diffusion of pyrene-labeled lipid (b) registered after addition of P178, the channel-forming domain of colicin E1, at different molar protein/lipid ratios.

Comparison of concentration dependences presented in Fig. 2 shows that P178 concentrations causing these processes are close to each other (protein/lipid ratio (1 : 2000)-(1 : 250) (CF release) and (1 : 6000)-(1 : 1000) (PyPC transfer)).

The kinetic parameters of the two processes are also similar. The most convenient parameter for description of CF release appeared to be $t_{1/2}$, the time required for half CF release, comparing to maximally possible leakage under these conditions (at $t = \infty$). It is seen that at equal protein/lipid ratios (1 : 1000) the $t_{1/2}$ parameter is 180 sec for CF leakage and 40 sec for flip-flop of PyPC.

For further substantiation of a common reason and mechanism of the two processes it was of interest to study their response to a certain effect. We have chosen the shift of the medium pH to alkaline region. According to our data [16], medium alkalization results in a sharp increase in the colicin E1-induced ion conductivity of planar bilayer lipid membrane (BLM) due to protonation of the His440 residue. We compared the effect of pH shift on two processes induced by colicin in liposomes: the dye release into solution and lipid flip-flop.

According to data of [11], colicin E1-induced calcein release from liposomes significantly decreases upon transition from pH 4.0 to more alkaline conditions. In line with this, in our experiments with CF leakage, addition of P178 to liposomes at pH 4.0 caused the release of the dye (Fig. 3a, curve *1* (see color insert) and Fig. 2a), whereas addition of P178 to liposomes at pH 6.0 had no such effect (Fig. 3a, curve *2*). It should be noted that shifting the pH from 4.0 to 6.0 in the absence of colicin provoked a stepwise increase in the fluorescence of the liposome suspension, which was probably caused by the known pH dependence of CF fluorescence. This increase, well noticeable in curve *2* of Fig. 3a, should be

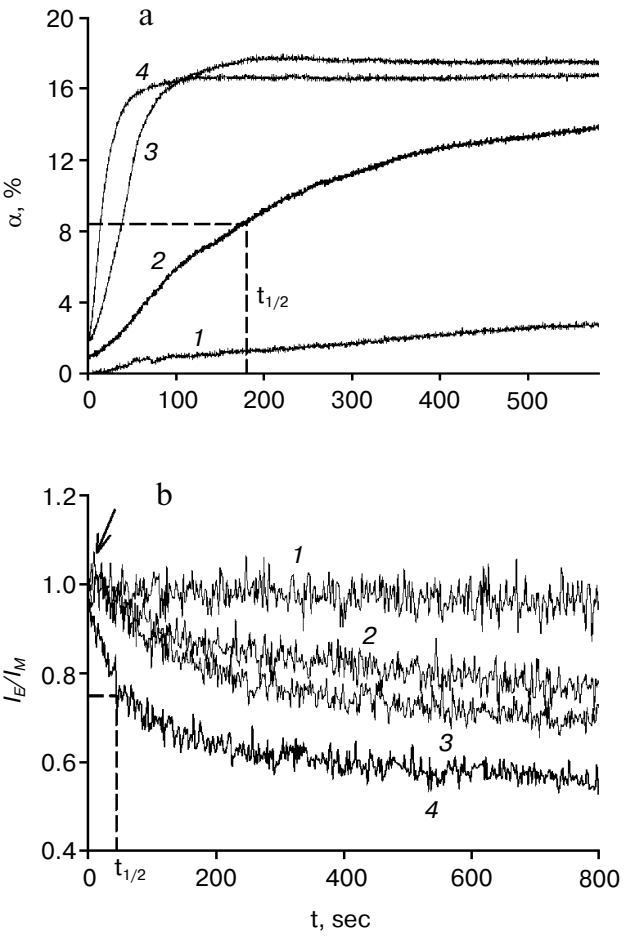

Fig. 2. Kinetic curves of fluorescent dye (carboxyfluorescein) release from liposomes (a) and flip-flop of pyrene-labeled lipid PyPC (b) registered after addition of P178, the channel-forming domain of colicin E1, at different protein/lipid molar ratios: a) time course of CF release from liposomes (DPhPC/DPhPG, 70/30 mol%) in response to 5 (*1*), 10 (*2*), 20 (*3*), and 40 nM (*4*) P178 at pH 4.0; lipid concentration 10 μM; b) time course of PyPC transmembrane diffusion in liposomes (DPhPC/DPhPG, 70/30 mol%) induced by addition (arrow) of 50 (*2*), 100 (*3*), and 250 nM (*4*) P178; curve *1* is a control; lipid concentration 300 μM. Time course of the process upon addition of 500 nM P178 coincides with curve *4*. The buffer solution contained 10 mM β-alanine and 0.12 M KCl, pH 4.0.

taken into account while comparing the colicin activity at pH 4.0 (Fig. 3a, curve *1*) and after the pH shift (Fig. 3a, curve *3*). As seen in the figure, the release of CF in response to P178 adsorbed at pH 4.0 is significantly stimulated by the alkalization of the medium: the extent of colicin-induced CF release was 48% after the pH shift and 8% at pH 4.0.

Similar results were obtained in investigation of flip-flop of lipids. Whereas at pH 4.0 P178 addition to liposomes resulted in significant decrease in I_E/I_M, indicative of PyPC redistribution from the external to internal monolayer (Fig. 3b, curve *1*), no induction of flip-flop was observed in response to P178 added to liposomes at pH 6.0 (Fig. 3b, curve *2*). However, if the protein was added to liposomes at pH 4.0 and then pH of the medium was shifted to 6.0, noticeable acceleration of flip-flop occurred (Fig. 3b, curve *3*).

Thus, pH shift from 4.0 to 6.0 results in stimulation of colicin E1-induced CF release from liposomes (a) and PyPC transmembrane diffusion (b).

Figure 3c shows the pH dependence of τ (the characteristic parameter of kinetics of the I_E/I_M change indicative of lipid flip-flop) which is achieved after addition of different KOH amounts to liposomes incubated at pH 4.0 with added P178, i.e. under conditions similar to those shown in curve *3* of Fig. 3b. It is seen that as the pH shift increases, τ decreases; in other words, the more pronounced is pH shift from 4.0 to alkaline region, the higher is acceleration of lipid flip-flop. The data show that even after medium alkalization the mechanism of colicin channel formation does not change, i.e. it directly involves lipid molecules.

Some comments should be made in description of experiments with colicin at different pH values concerning the effect of medium acidity on the colicin binding to membrane, which undoubtedly contributes to pH dependence of colicin activity. The peculiarity of colicin E1 is that after initial binding to the membrane by electrostatic interactions (efficient binding is observed at acidic pH [17, 18]), later the main role belongs to hydrophobic interactions between protein molecules and lipid bilayer. This in particular results in the fact that even under conditions when electrostatic interactions weaken (for example, in the case of pH shift towards alkaline values), colicin is not desorbed from the membrane. Such a result was obtained by Zakharov et al. [18] in experiments on determination of colicin binding to liposomes containing fluorescence quencher in the form of trinitrophenyl group (TNP) and confirmed by us in this work by measurement of colicin binding to brominated liposomes. The difference between these experiments is that the use of brominated liposomes (a quencher which is localized practically in the center of the bilayer) allows one to reveal significant immersion of the colicin α-helices in BLM, whereas the use of the system with TNP demonstrates the interaction of colicin with the membrane surface.

We measured P178 binding to the membrane from quenching of tryptophan fluorescence in colicin by liposomes incorporating brominated phospholipids (Fig. 4a). The comparison of curves *1* and *2* shows that addition of liposomes with brominated lipids to buffer solution containing P178 results in significant drop in tryptophan fluorescence, which is indicative of protein binding to liposomes [2, 10, 19, 20]. Curve *3* shows that subsequent pH shift (4→7) causes practically no change in the tryptophan fluorescence of P178. This means that in the case of alkalization the protein remains incorporated in the lipid bilayer.

Comparison of curves *1* and *2* of Fig. 4b shows that addition of liposomes containing brominated lipids to

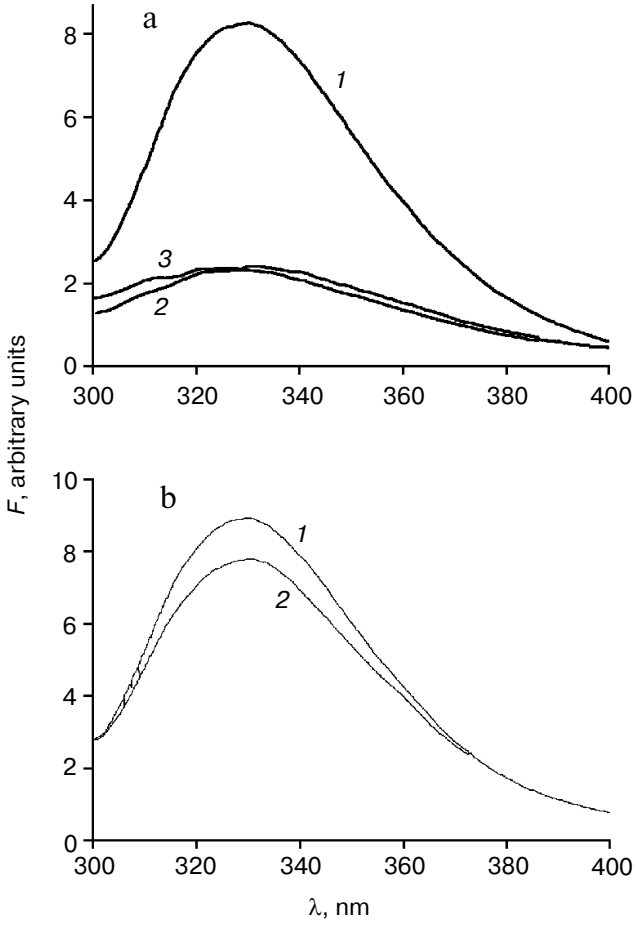

Fig. 4. Effect of pH on P178 binding to liposomes measured from tryptophan fluorescence quenching by brominated lipids. a) Alkalization effect on P178 binding to BLM. Fluorescence spectra were measured after successive additions of 500 nM P178 (curve *1*), 400 μM liposomes formed of Br-PC/DPhPG in 7 : 3 molar ratio (curve *2*), and titrated in advance KOH amount necessary to shift buffer pH to 7.0 (curve *3*). The buffer solution contained 10 mM β-alanine, 10 mM MES, 10 mM Tris, 0.12 M KCl, pH 4.0. b) P178 binding to BLM at pH 7.0. Fluorescence spectra were measured after successive additions of 500 nM P178 (curve *1*) and 400 μM Br-PC/DPhPG (7 : 3) liposomes (curve *2*). Excitation wavelength, 282 nm.

P178 solution at pH 7.0 did not result in a noticeable drop in tryptophan fluorescence of the protein, thus showing that colicin effectively binds to membrane only at acid pH, whereas at neutral pH its binding to membrane is extremely low.

DISCUSSION

The data obtained in this work are indicative of correlation of two processes caused in lipid membranes by colicin E1: the process of pore formation detected by the fluorescent dye release from liposomes and transmembrane lipid diffusion measured by decrease in excimer fluorescence of pyrene attached to phosphatidylcholine. Such correlation confirms the validity of the pore toroidal model (proteolipidic) for the colicin channel structure. Earlier [21] similar correlation was shown on liposomes for pore formation and stimulation of pyrene-labeled lipid flip-flop caused by proapoptotic protein Bax (combined with t-Bid protein), which together with dependence of these processes on the membrane curvature is considered as proof of the pore toroidal structure induced by Bax protein [22], whose channel-forming domain is structurally related to the corresponding domain of pore-forming colicins [23]. Note that the significant correlation of time and concentration dependences of calcein release from liposomes and flip-flop of NBD-labeled lipids obtained for magainin [5], along with data of crystallography [7, 24], became the basis for a model of protein–lipid toroidal pore supposing direct involvement of lipid head groups in formation of the conducting structure in the membrane.

It should be noted that $t_{1/2}$ in the case of colicin E1-stimulated flip-flop is significantly lower than in the case of CF release, and active protein concentrations are also lower (Fig. 2). Within the framework of the supposed scheme of the process (Fig. 5), this observation suggests that upon colicin interaction with membrane, the lipid flip-flop is initiated already at the initial stages of protein

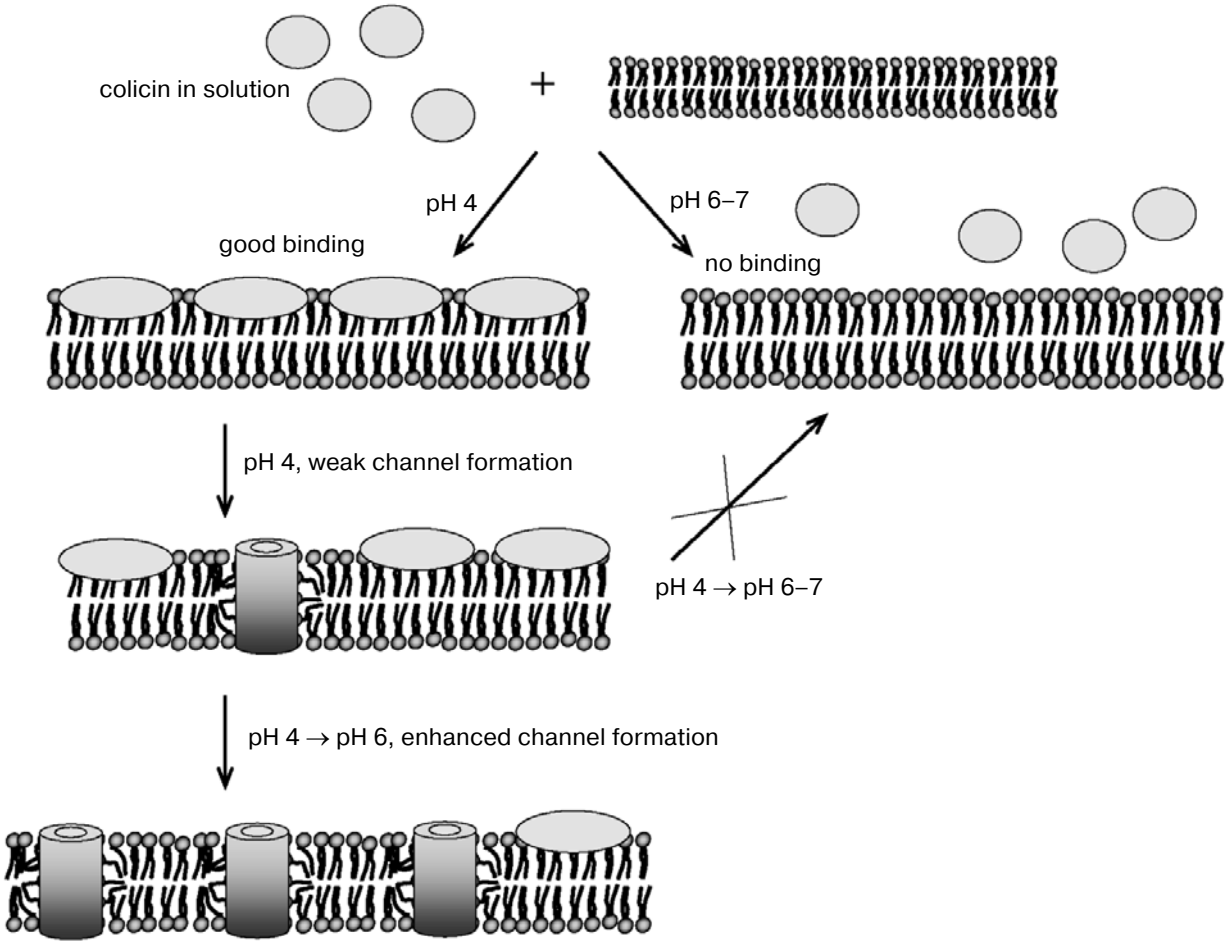

Fig. 5. Scheme of the colicin E1 channel-forming domain interaction with membrane at different pH values. Colicin efficiently binds to the membrane at acidic pH. However, channel formation in this case is hampered. Upon subsequent alkalization of the medium the colicin channel formation is significantly enhanced, and no protein desorption from the membrane surface is observed.

incorporation into bilayer, while pore formation is the final stage of the process.

As noted previously [3], four transmembrane α-helices of the colicin channel-forming domain are obviously not sufficient for formation of pores over 12 Å in diameter; therefore, lipid molecules should be involved in channel formation. The pH shift-activated process of colicin-induced release from liposomes of such large dye as carboxyfluorescein is indicative of big size of the formed pores, which is well explained by involvement of head groups of lipid molecules in formation of the conducting state.

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