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The mode of action of *Vibrio cholerae* cytolysin. The influences on both erythrocytes and planar lipid bilayers

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The interaction with erythrocytes of cholera cytolysin (CC) obtained from a non-01 *Vibrio cholerae* strain results in the osmotic rupture of target cells upon formation by CC of the waterfilled pores in their membranes. The aggregation of several toxin monomers is required for the formation of one CC channel with a radius of 0.9–1.0 nm. The investigations using planar bilayer lipid membranes suggest that the CC-induced pore is an interprotein anion selective channel carrying a fixed positive charge. The role of the charge was supported by the influence of pH on the selectivity, single conductance and voltage gating of the CC channels. The ability of the CC to modify both model and natural membranes has a maximum at pH 6.0–7.0. It was found that CC channels insert into the membrane asymmetrically. The effect of proteolytic treatment of the channel by papain also indicates that the two entrances of the channel protrude from the plane of the membrane into the solution for different distances. It is proposed that the biological effects of the non-01 *V. cholera* cytolysin are based on its channel-forming activity.

Introduction

Historically, *Vibrio cholerae* strains that lack the 01 antigen (non-01 *V. cholerae*) were believed to be non-pathogenic. It is now known, however, that these strains are able to cause diarrhea. Some non-01 V. cholerae strains have been found to produce a thermolabile enterotoxin that is similar to cholera enterotoxin [1–3]. But non-01 *V. cholerae* strains that do not produce cholera-like enterotoxin also cause illness [4–7]. This fact has led to a search for other pathogenic mechanisms. Based on the fact that most of the non-01 *V. cholerae* strains produce a large amount of hemolysin [8,9] it was supposed that the hemolysin is an enterotoxic factor that is responsible for the non-01 *V. cholerae* gastroenteritis. Recently a non-01 *V. cholerae* hemolysin was purified and characterized by several researchers from different strains as a thermolabile lethal cyto- and entero-toxic protein which is related to El Tor hemolysin [10–12]. Thus the enteropathogenic action of the cytolysin seems to be correlated with hemolysis, but the primary mechanism of erythrocytes damage has not been studied.

Our studies in this area are directed at defining the mechanism of action of non-01 *V. cholerae* cytolysin (CC). We provide evidence that the toxin forms large waterfilled pores with radii of approx. 0.9–1.0 nm in both biological (erythrocyte) membranes and artificial planar lipid bilayers. The defined pores are anion selective channels that are formed by more than one molecule of CC. The obtained data point out that the CC-induced pores are surrounded by proteins and entrances of the channel are distant from the membrane surfaces. Our results indicate that channel-forming activity is the property of the CC which plays a key role in the toxin mode of action.

Materials and Methods

Bacterial strain. The cholera cytolysin (CC) was obtained by one of us (Zitzer, A.) from the culture supernatant of non-01 *Vibrio cholerae* strain which was isolated from Kapchagai reservoir waters 8.07.81, and was maintained by lyophilization since that time. The CC was purified by complex liquid chromatography methods (will be published separately). Used preparation of the CC was homogeneous by sodium dodecyl

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Abbreviations: CC, non-01 *Vibrio cholerae* cytolysin; BLM, planar bilayer lipid membranes; PC, phosphatidylcholine; PS, phosphatidylserine; PEG, poly(ethylene glycol); Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; NT buffer, 150 mM NaCl, 5 mM Tris-HCl (pH 7.5); HU, hemolytic unit.

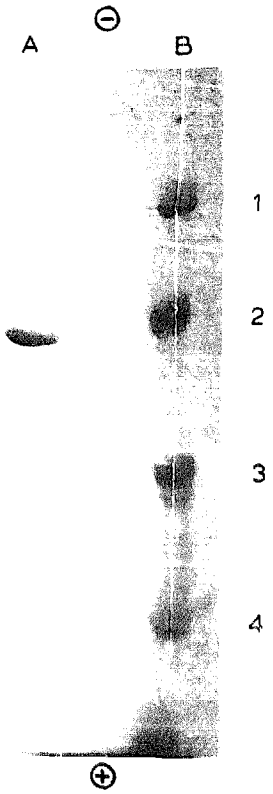

Fig. 1. Sodium dodecyl sulfate-polyacrylamide gel (SDS-PAAG) slab electrophoresis of non-01 *V. cholerae* cytolysin (lane A). Marker proteins (lane B) molecular mass: 1, phosphorylase *b* (94 kDa); 2, bovine serum albumin (68 kDa); 3, ovalbumin (43 kDa); 4, carbonic anhydrase (30 kDa).

sulfate polyacrylamide gel (SDS-PAAG) slab electrophoresis (Fig. 1).

Protein chemistry. The molecular weight of the purified toxin was determined by SDS-PAAG slab electrophoresis. Protein concentrations in solution were determined by the Bradford technique [13], determination of the isoelectric point (p*I*) was done according to Ref. 14. Amino acid analysis was done by Dr. Korneev (Institute of Bioorganic Chemistry, Tashkent) and was confirmed by Dr. Teleginskaiy (Institute of Bioorganic Chemistry, Moskow).

Hemolytic activity assay. Rabbit erythrocytes were washed with 150 mM NaCl, 5 mM Tris-HCl, pH 7.5 (NT buffer) four to five times and were adjusted to give a concentration of 4% (v/v). The toxin diluted in the same buffer (0.5 ml) was added to 0.5 ml of the erythrocyte suspension and vigourously mixed. This mixture was incubated at 37°C for 60 min and then was centrifuged at 3000 × *g* for 3 min. The optical density of the 4-fold-diluted supernatant was measured at 540 nm. The percentage of hemolysis was calculated with erythrocytes lysed by distiled water taken as 100%. One hemolytic unit (HU) was defined as the amount of toxin causing 50% hemolysis of 1 ml of 2% erythrocytes suspension after 60 min at 37°C.

Potassium leakage measurement. To measure the kinetics of CC-induced leakage of K ions from human erythrocytes at 37°C we picked out 100 μl of the 2% suspension of red blood cells at 5, 10, 20, 40 and 60 min after addition of the toxin to a final concentration 0.25 μg/ml. Each sample was diluted to 1 ml with cold NT-buffer and rapidly sedimented. Concentrations of K^+ in the supernatants were measured by a flame photometer.

Planar bilayer membranes. Formation and properties. Planar bilayer lipid membranes (BLM) were formed at room temperature by the union of two monolayers [15]. The following lipids were used: common fraction of bovine brain phospholipids, phosphatidylcholine (PC) and phosphatidylserine (PS). Monolayers from a 1 mg/ml solution of these lipids in n-hexane were spread on the water surface of experimental chamber and after evaporation of the solvent a membrane was formed on a hole in a 20 μm thick Teflon partition separating two buffered salt solutions (2 ml). The hole (0.2–0.4 mm in diameter) was pretreated with a 1:20 (v/v) solution of hexadecane in n-hexane. Experiments were done under voltage-clamp conditions with a single pair of Ag/AgCl electrodes that made electrical contact with solutions in the compartments through 3 M KCl agar bridges. The membrane conductance, G was defined as $G = I/V$, where I was the current flowing through the membrane and V was the potential of the cis compartment. The trans compartment was connected to the virtual ground and voltage signs are referred to it. After the membrane was completely formed and stabilized, a portion of the toxin was added to one compartment to concentrations ranging from 0.125 μg/ml to 2.5 μg/ml. In separate experiments the CC was added by application of stock solution on the membrane. Cation transference number (t_+) was calculated from the slope of dependence of zero-current potential versus KCl concentration which was changed by consecutive addition of 3.5 M KCl into the trans compartment of experimental chamber while the cis one contained unchanged 100 mM KCl solution.

Channel-sizing experiments. To measure the radius of the water pores induced by the CC in erythrocytes membranes we used some nonelectrolytes, which were previously added into NT solution to a final concentration (mM): glucose, sucrose poly(ethylene glycol) (PEG) 400 (40), PEG 1000 (33), PEG 1500 (27.5), PEG 2000 (23), PEG 6000 (12). Increasing of NT solution osmolarity after such additions were the same and equal to 40 milliosmoles per litre as measured by freezing point depression with osmometer OMKA 1C-01. This osmolarity value was close to that for hemoglobin in erythrocytes [16]. 0.5 ml of the CC diluted with the desired nonelectrolyte solution was added to 0.5 ml of a 4% suspension of rabbit erythrocytes and the mixture was incubated at 37°C for 60 min. The effective radius of

toxin-induced water pores into erythrocyte membranes was determined equal to the minimal hydrodynamic radius of such nonelectrolyte molecules which completely protected red blood cells to cytotoxin action.

To estimate the radii of pores induced by the CC in BLM we used aqueous solutions of different nonelectrolytes bathing bilayers. Usually, solutions, 2 ml on each side, contained 100 mM KCl, 5 mM Tris-citrate, one of the nonelectrolytes (20%, w/v), pH 7.0. The values of both the single-channel conductance and the solution conductivity in each medium were measured.

Chemicals. We used poly(ethylene glycol) (PEG) with average molecular mass (Da); 400 (Schuchardt, Germany); 300, 1000, 1500, 2000, 4000, 6000 (Loba Chemie, Austria). All nonelectrolytes were additionally purified by anion-exchange chromatography by using anionit AV-21. Hydrodynamic radii of nonelectrolytes were measured in our laboratory using the viscosimetrical method described earlier [17]. The conductivity of each buffer solution was measured with a Radelkis OK 102/1 conductometer at 25°C. Common fractions of bovine brain phospholipids, PC and PS were prepared according to Ref. 18, packed in ampuls and stored at −20°C for two months without change in its properties. Cholesterol was purchased from Sigma (USA), dithiothreitol from Koch-Light (UK), papain from Loba Chemie (Austria).

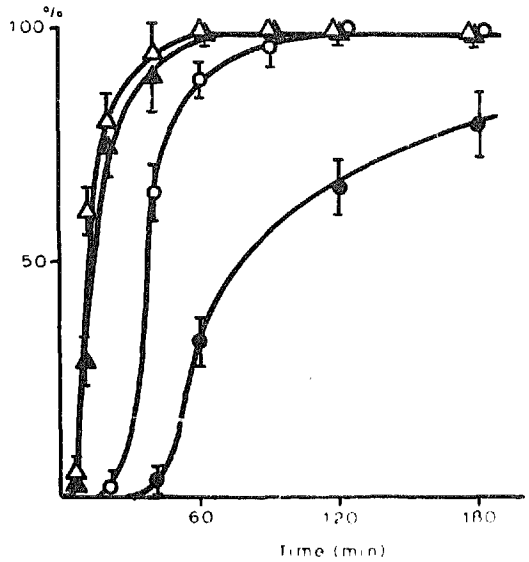

Fig. 2. The time-dependent leakage of K (△) and hemolysis (○) of human erythrocytes treated by CC. The toxin was added to 10 ml of 2% suspension of human erythrocytes in a final concentration 0.5 μg/ml (open symbol) or 0.25 μg/ml (filled symbol). After addition of the toxin a portion (100 μl) of the erythrocyte suspension was taken in desired time period. Then each of samples was diluted up to 1 ml with NT buffer and fast sedimented. The both optical density (at 540 nm) and concentration of K (by flame photometer) were measured. The percentage of hemolysis and K leakage was calculated with 100% lysed erythrocytes by distilled water. The presented value are the means of 3–6 experiments.

Results

The mechanism of lysis of red blood cells treated by the CC and the size of the CC-induced water pores in erythrocyte membranes

Cholera cytolysin (CC) interacts with erythrocytes and effectively lyses them [11,19]. However, the primary mechanism of erythrocyte damage by CC is not known. The following simple experiments allowed us to reach a conclusion about the mechanism. First, a time-dependent lysis of erythrocytes treated by CC (Fig. 2) demonstrated a rapid K^+ efflux that was followed by the release of hemoglobin, i.e., hemolysis. At a higher concentration of the toxin, a shorter time interval between the efflux of K^+ and the release of hemoglobin was observed, but the sequence of the events was not changed. This fact indicated that the hydrodynamic diameters of hemoglobin molecules are larger than the sizes of CC lesions. So the hemoglobin can not pass through the lesions. Release of the hemoglobin is, probably, a result of osmotic rupture of the cells. This suggestion was supported by finding that uncharged macromolecules, such as poly(ethylene glycol) (PEG) 4000, in the extracellular medium protected cells from lysis. The protection of erythrocytes by PEG 4000 was complete even if the cytolysin concentration was raised to 5 μg/ml (concentration of CC induced 50% lysis of rabbit erythrocytes was equal to 0.1 μg/ml). Therefore the size of pores formed by the CC in the membrane actually are smaller than the hydrodynamic diameter of hemoglobin. Moreover, we can conclude that the CC-induced lesions obviously are homogeneous, i.e., the size of water pores are negligible changed at various concentration of the toxin. The effect of PEG 4000 was not due to interference between nonelectrolytes and toxin molecules binding to erythrocyte membranes. Substitution of the nonelectrolyte-containing media by fresh NT buffer without nonelectrolytes led to irreversible hemolysis. The data indicate that CC-induced hemolysis occurs by an osmotic mechanism similar to the mechanism of action of other bacterial toxins [20–23].

The inhibitory effect of nonelectrolytes depended on hydrodynamic radius of their molecules (Fig. 3). We found that macromolecules of nonelectrolytes, such as PEG 1000, PEG 2000 and higher completely inhibit the CC induced lysis of the red blood cells, but molecules PEG 300, sucrose and other small hydrocarbons and alcohols did not. The minimal hydrodynamic radius of nonelectrolyte molecules which completely inhibited the CC-induced cell lysis was close to 0.95 nm. Therefore we can conclude that in the membranes of target cells the CC formed pores with average radii of about 0.9–1.0 nm.

Influence of the CC on planar bilayer lipid membrane properties

Investigations which we carried out on planar bilayer lipid membranes (BLM) provided conclusive evidence in support of the pore concept. The addition of CC to voltage-clamped lipid bilayers led to stepwise increase of the BLM conductance (Fig. 4). It indicated the formation of ionic channels in the membrane. The conductance value for the unitary event was calculated and summarised in the cumulative histogram shown in the inset of Fig. 4. To investigate the selectivity of the channels we measured the transmembrane potential when the membrane divided solutions with different concentrations of the same electrolyte. The results indicated that the channels were about 5-times as permeable to chloride as to monovalent cations ($t_+ = 0.18$ at 100 mM KCl pH 7.5), with practically no selectivity for Na^+ over K^+, since in biionic experiments (100 mM KCl/100 mM NaCl) the transmembrane potential was usual less than 3 mV. Thus the CC actually changes the properties of membranes by the formation anion selective waterfilled channel structures.

To establish whether one molecule of the cytolysin can form the ionic channel into the membranes we have studied various dose-response dependences. Fig. 5 shows that the slopes of the dependences of the maximum pore formation rate, the steady-state number of channel in BLM and the level of hemolysis of rabbit red blood cells on protein concentration in double-logarithmic plots reached to 2. These data doubtless indicate that an aggregation mechanism does indeed operate to produce the CC channels and that more than one molecule of the toxin takes part in the formation of the waterfilled pore in artificial, as well as in biological, membranes.

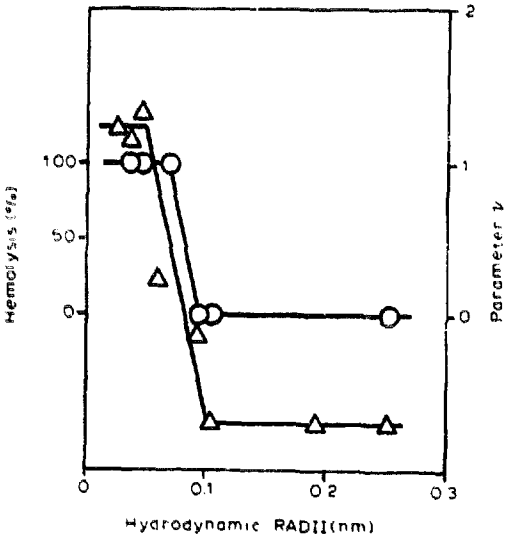

Fig. 3. The molecular size dependent effect of non-electrolytes both on hemolysis of erythrocytes and parameter ν. Percent of CC-induced lysis of 2% rabbit erythrocyte suspension contained nonelectrolytes (○); left vertical scale. The toxin was added in a final concentration 0.5 μg/ml. The values represented here are means of five separate experiments. The permeability parameter ν of each nonelectrolyte (△) was calculated as described in Results; right vertical scale. Composition of the solutions containing nonelectrolytes are described in Materials and Methods. The hydrodynamical radii of non-electrolytes measured by viscosimetrical method were the following (nm): ethylene glycol, 0.26 ± 0.01; glucose, 0.37 ± 0.02; sucrose, 0.47 ± 0.01; poly(ethylene glycol) (PEG) 300, 0.6 ± 0.02; PEG 400, 0.7 ± 0.03; PEG 1000, 0.94 ± 0.06; PEG 1500, 1.05 ± 0.01; PEG 2000, 1.22 ± 0.01; PEG 4000, 1.92 ± 0.03; PEG 600, 2.5 ± 0.03.

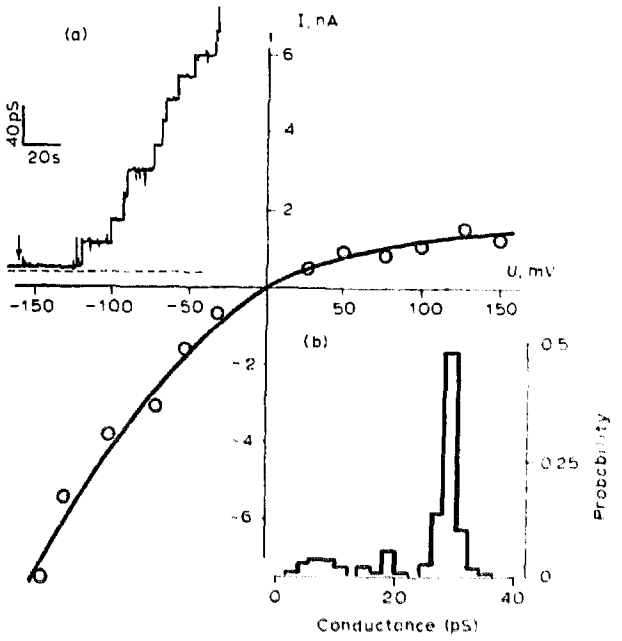

Fig. 4. Current-voltage characteristic of channels induced by *V. cholerae* cytolysin in planar bilayers. BLM was formed as described [15] from common fraction of bovine brain phospholipids. Planar bilayers were bathed with 150 mM NaCl, 5 mM Tris-citrate, pH 7.5 solution. The trans compartment was connected to the virtual ground. Triangular voltage pulses of 10 Hz frequency (line) or long-lasting voltage pulses (20–30 s duration; ○) were applied to the cis compartment. The toxin (5 μl) was applied on the cis side of BLM from stock solution (100 μg/ml). Inset: (a) current steps after addition of the cytolysin to cis side of BLM clamped at −50 mV; each one is due to the opening of a new ionic channel into the membrane. The dashed line indicates a current of zero while the arrow points at the moment of addition of the toxin (0.25 μg). Conductance and time scales are given in the figure; (b) the histogram representing the probability P to observe steps of the CC-channels conductance like that in the current trace; 123 steps, bin width 5 pS.

The size of the CC channel water pores in BLM

To measure the effective radius of the CC channel in BLM we have used the method described in detail earlier [17]. This method has been successfully applied to determine the sizes of channels induced by whole α-staphylotoxin [24] and its tryptic fragment [25], latrotoxin [26] and B-subunit of cholera toxin [27]. It had been developed for waterfilled pores. For these pores the direct correlation between the solution conductivity and single-channel conductance has to be observed at

least for short time intervals. In our case, the hemolysis experiments indicate that the CC channels are, perhaps, large enough waterfilled pores. To check this possibility we studied the behaviour of the channel at various KCl concentration in bathing BLM media. The mean conductance of the CC channel increased when the activity of KCl solution was increased (Fig. 6). The same influence of KCl activity on the solution conductivity indicates a linear relationship between these two variables (the coefficient of correlation is 0.993). Thus the conditions noted above were almost realized and we could use the noted above method.

To estimate the radius of water pores induced by CC in BLM we studied the changes of both the solution conductivity and the channel conductance resulting from the addition of different non-electrolytes to the aqueous solution bathing the bilayer. For analysis of the changes of these values and the following determination the pore size of the ionic channels we have to use a permeability parameter, ν, which was calculated as:

$$\nu = \frac{(g - g')}{g} : \frac{(H - H')}{H}$$

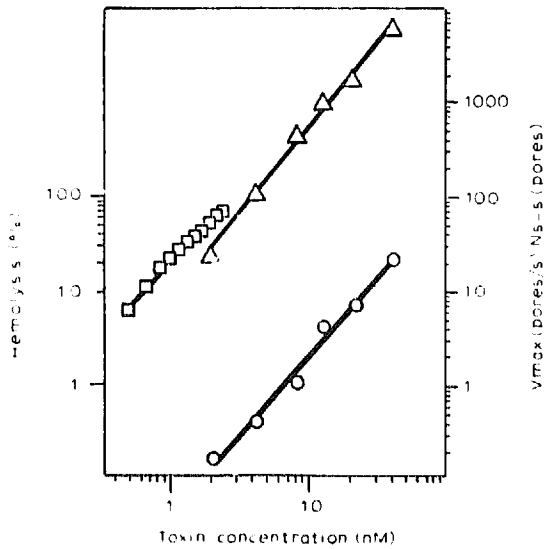

Fig. 6. Double-logarithmic plot of single-channel conductance and solution conductivity against activity of KCl. The values of single CC channels conductance (△) were obtained from steps of current like that in Fig. 4. The solutions used with different concentration of KCl, were buffered by 5 mM Tris-citrate (pH 6.0). The total number of events (current steps) is about 650. The slope of the least-squares line is 1.1. The electrical conductivity of solutions (○) was measured using a conductometer at 25°C. The slope of the least-squares line is 1.0.

Fig. 5. Double-logarithmic plot of the maximum pore formation rate, steady-state number of channels and percent of hemolysis against the toxin concentration. The slopes of the maximum pore formation rate (○) and steady-state number of channels (△) are 1.9 and 1.7, respectively. BLM clamped at −20 mV was bathed with 150 mM NaCl, 5 mM Tris-citrate, pH 6.0 solution. Toxin in a different concentration was added into the cis compartment. The maximum pore formation rate, V_{max} and the steady-state number of pores in the membrane, N_{s-s} were calculated as

$$V_{max} = [(dI/dt)_{max}/G_0] \cdot U;\ N_{s-s} = [I_{s-s}/G_0]\ U,$$

where U is the constant applied voltage and G_0 the single-channel conductance value. The dependence of rabbit red blood cells hemolysis against toxin concentration (□) have a slope 1.5. All of the straight lines are least-squares fit. Other conditions of experiment are described in Materials and Methods.

where H and g are the electrical conductivity of 100 mM KCl and the channel conductance in the same solution, respectively;

H' and g' are the electrical conductivity of 100 mM KCl solution containing 20% nonelectrolyte and channel conductance in the same solution, respectively.

Determining the permeability parameter for each nonelectrolyte we found that it depended on the effective hydrodynamic radius of their molecules (Fig. 3). So nonelectrolytes with small hydrodynamic radii (such as glycerin, glucose and sucrose) changed the conductance of the ionic channel proportionally to the decrease of the solution conductivity and the numerical values of the permeability parameter (ν) were about 1, i.e., the CC channels are well permeable for molecules of such nonelectrolytes. When the size of used nonelectrolyte molecules increased the values of the parameter ν decreased from 1 to 0. Hence the concentration of nonelectrolyte molecules in the water pore of the channel was less than that in the bulk solution. It appeared, obviously, when the size of the nonelectrolyte molecules approached the effective size of the CC-induced water pore. Finally, the values of parameter ν for impermeant large non-electrolytes formed the lowest horizontal branch of the dependence ν on the hydrodynamic radius of nonelectrolyte molecules (Fig. 3). In this case, parameter ν was constant and had a slightly negative value.

It should be noted that after addition of the impermeant nonelectrolytes into the bathing solution the conductance of the CC channels was increased in compare with conductance of the channels measured in the

same buffer but without nonelectrolyte although the conductivity of the solutions was decreased drastically. In common, a noted increase of the CC-channels conductance and, respectively, a negative value of parameter ν (for the used impermeant large molecules) result because the activity of ions in nonelectrolyte-containing solutions was about 30% larger than that in the solutions without PEG. The detailed analysis of that and other effects of nonelectrolytes was done earlier [17]. According to the method we supposed that the effective radius of the CC-channel water pore is located at the point of transition from the linear falling part to the lower horizontal branch of the permeability parameter dependence on the hydrodynamic radius of nonelectrolytes (Fig. 3). In this way we established that the effective radius of the ionic channel water pore induced by CC in planar bilayer membranes was equal to 0.9–1.0 nm.

These data point out a practical identity fo the effective size of water pore induced by CC in both BLM and erythrocyte membranes. Obviously, the structure of these water pores in the two types of membrane also should be close to each other.

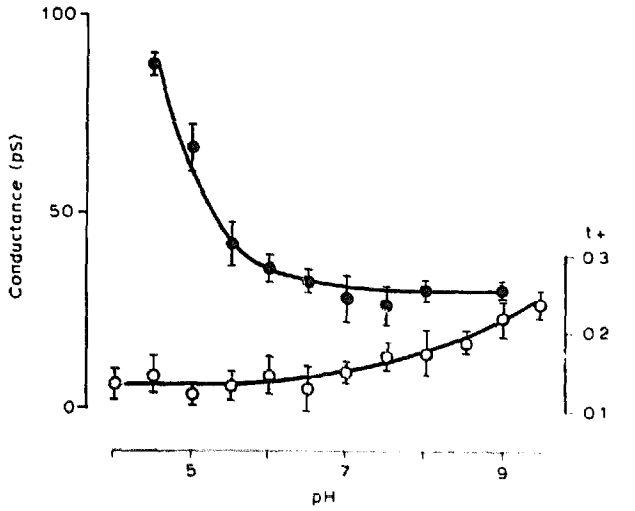

Fig. 7. Effect of pH on the selectivity and conductance of channels induced by cytolysin in BLM. Conductances of single channels (●) were measured in 150 mM NaCl, 5 mM Tris-citrate at desired value of pH; left vertical scale. The number of events was 40 to 150 for each value of conductance which were obtained as described in the legend to fig. 2. Right: the cation-anion selectivity (t_+) of CC-channels (○) was calculated from measurements of zero current potential as described in Materials and Methods.

The influence of lipid bilayer composition on the properties of CC channels

To comprehend the ion-conductive structure of any ionic channel it is necessary to clarify the contribution of both the protein and the lipid to the formation of this structure. With this aim we have studied the pH dependence of cation-anion selectivity of the CC-modified BLM. We have found that the intrinsic anion selectivity of the CC channels was remained practically complete even if the CC channels were incorporated in the negative charged BLM at all used pH of water solution (Fig. 7; Table II). This fact indicates that a water pores of the channels are mainly surrounded by protein molecules but not lipids.

This conclusion was supported by comparison of the single CC channel conductance inserted in both pure PC and mixed PC/PS bilayers. The negative surface potential of the PC/PS bilayer should attract cations and, accordingly, repeal anions. Now, if the pore entrance is located near the membrane surface, the conductance of the CC-channel incorporated in the uncharged (PC) or in negative charged (PC/PS) BLM should be strongly different. Taking into account the anion selectivity of the cytolysin channel we could expect a considerable decrease in the single conductance of the channel inserted in PC/PS bilayers in comparison with that in pure PC membranes. However, we have established that the mean channel conductances were practically equal to each other (27.8 ± 2.1 pS and 25.6 ± 2.3 pS for PC and PC/PS bilayers, respectively) under identical experimental conditions (100 mM KCl, 10 mM Tris-HCl, pH 7.0). This, doubtless, indicates that the pore entrances are located far away from the surface of the membrane.

The location of CC-channel in BLM

The current–voltage characteristic (CVC) of the multichannels membrane modified by the CC at pH 7.5 was sharply asymmetric. Such result was obtained when we applied as the triangular voltage pulses of 10 Hz frequency as long-lasting voltage pulses (Fig. 4). The CVC of a single CC-channel under identical conditions had also the same shape. Hence this is indeed an intrinsic property of the open CC-channel.

In addition the shape of CVC of the CC channels allow us to propose that the channels are asymmetrically inserted into the bilayer. To check this idea we used asymmetrical bilayers, where one monolayer was made from pure PC and the other from PC/PS (1 : 1) mixture. The toxin was applied to the cis-side of BLM and then the instantaneous CVC has been measured. To compare the various CVCs and to measure the contribution of the lipid monolayers to the determination of the CC-channel properties we have calculated some parameters of CVC. So the relationship between the current through a single channel at +100 mV and its value at −100 mV is taken as a quantitative parameter of CVC asymmetry (A), and the linearity parameter corresponds to the relationship of the current value measured at 100 mV and that obtained by linear extrapolation of the current value at 20 mV (L). The results of the set of experiments are presented in Table I. It was found that the CVC shape of the CC channels depended on the lipid composition of the trans-mono-

layer of BLM. When the CC was added to the PC monolayer of BLM the influence of trans charge monolayer (PS/PC) on the parameters of CVC was more ponderable. This means that the entrances of the channel protrude from the plane of the membrane into the solution for different distances. If we call the CC-channel entrance which protrudes from the plane of the membrane into the side of cytolysin addition the 'Exe' entrance and the entrance on the other side the 'In' entrance, than we can conclude that the 'Exe' entrance of the channel, obviously, is placed farther from the surface of the polar lipid heads than the 'In' entrance.

The effect of proteolytic enzymes on the properties of the CC channel

Further information about the location of the cytolysin channel in the membrane was gained by the limited proteolysis of the toxin upon formation of the ion-conductive structure of the CC channel. It was found that the proteolytic enzyme papain influenced the cation-anion selectivity of the CC modified bilayer. This set of experiments was done as follows. First, the cytolysin was added to the bathing BLM solution on the cis side of the preformed BLM. When the membrane conductance has reached the final steady state, the papain (final concentration 1 mg/ml) was injected in either both sides or only one side of the experimental chamber. After exposition for 60 min at 25°C the bathing solution was substituted with the initial buffer. Finally, the selectivity and CVC of the multichannel BLM treated with the enzyme was measured. The results are shown in Table II. It was established that the treatment of the definitive channel structure by papain led to the decrease of its selectivity. Besides the influence on the selectivity of the CC-channel, the treatment with papain also led to an 2–3-fold decrease in steady-state membrane conductance. Surprisingly, the shape of CVC changed slightly.

The decrease in selectivity, but to a smaller degree, was observed if we added the enzyme to the cis (the toxin addition compartment) side of the experimental chamber only. The addition of the enzyme to the opposite compartment of the chamber did not change the selectivity of the CC channels. Hence only the 'Exe' entrance of the CC channel is available to proteolytic enzyme. These findings allow us to conclude that the 'Exe' entrance of the CC pore is placed farther from the plane of the membrane than the 'In' entrance.

TABLE I

The influence of lipid composition on the shape of current–voltage characteristics (CVC) of the CC channels

The bilayers were formed by the union of two monolayers of which the lipid composition is noted in the table. The CVCs were obtained by applying long-lasting voltage pulses to the CC-modified membrane. The toxin was added to the cis compartment, while the trans compartment was connected to the virtual ground. The parameters which quantitatively characterize asymmetry (A) and linearity (L) of the CVC shape were calculated as described in Results. Experimental conditions were: 100 mM KCl, 10 mM Tris-HCl (pH 7.0). 9–20 experiments were carried out to obtain each value of parameters.

Parameters of CVCs	Symmetrical bilayer		Asymmetrical bilayer	
	PC-trans PC-cis	PS/PC-trans PS/PC-cis	PC-trans PS/PC-cis	PS/PC-trans PC-cis
$-L$	1.62±0.06	1.86±0.09	1.68±0.08	1.85±0.06
$+L$	0.70±0.05	0.63±0.06	0.72±0.04	0.62±0.05
A	2.95±0.16	4.12±0.56	3.25±0.13	3.89±0.12

TABLE II

The influence of enzymatic treating of the CC channels on their selectivity

The BLM were formed from a PS/PC mixture. The CC was added by applying 5 μl of stock toxin solution (0.1 mg/ml) on the cis side of the BLM. Papain (1.6 mg) was injected into the bathing solution (1.6 ml): 100 mM KCl, 10 mM Tris-HCl (pH 7.0). The selectivity of the CC channels under these conditions are presented here using the cation transference number (t_+), calculated from the slope of dependence of zero-current potential versus KCl concentration. The values of the zero-current potential were measured after every addition of 3.5 M KCl into the trans compartment of the experimental chamber while the cis one contained unchanged 100 mM KCl solution. 3–5 successful experiments were carried out to calculate the value of the channel selectivity. The course of the experiments was described in Results.

Enzyme (papain):	Absent	Cis/trans	Cis	Trans
Selectivity (t_+)	0.18±0.02	0.36±0.15	0.29±0.04	0.18±0.03

Discussion

The place of the cytolysin among other hemolysin secreted by non-01 Vibrio cholerae

We presented the results of our study of the effects of toxin which was secreted by non-01 *V. cholerae* strains on membranes. It was found that the toxin effectively lysed erythrocytes from various species. The relative sensitivity of erythrocytes to this toxin was in the following order: rabbit > guinea pig > rat > sheep > human = chicken > pigeon = turkey > frog. The concentration of the CC causing 50% lysis of rabbit red blood cells was 0.1 μg/ml. The most resistant to the action of the toxin were erythrocytes of the frog (1.6 μg/ml). The sensitivities of the other tested cells to CC differ not greatly (0.1–0.5 μg/ml). So the studied toxin is a hemolysin. Moreover, as was shown by Zitzer (personal communication), the purified toxin also was able to damage different lines of mammalian cells and possessed other biological properties. It had a lethal activity to white mice at i.v. injection; induced the accumulation of mucous fluid in the small intestine and darkly coloured fluid in the cecum of rabbits.

All of the biological activities of the CC completely abolished after incubation of the toxin solution at 100°C for 1 min. The activities were not altered by addition of cholesterol or dithiothreitol into toxin solution. The CC preparation didn't show any phospholipase activity.

All the properties noted above were not due to some contaminates but due to a protein with a molecular weight of about 60000 and p*I* 6.2. SDS-PAAG electrophoresis showed that purified CC migrated as a single band with that molecular weight (Fig. 1). Thus like a hemolysin produced by *V. cholerae* biotype El Tor this cytolysin was thermolabile, hemolytic and lethal for mice. Generally, several toxins secreted by *V. cholerae* may be gathered into one group of El Tor-like hemolysins. Beside the general properties noted above these toxins differ from each other by their molecular weights and isoelectric points. So the cytolysin obtained by McCardell and Madden [11] had a molecular weight equal to 52711 and p*I* 8.65; El Tor hemolysin purified by Honda and Finkelstein [28] was characterized as a protein with M_r 20000 and hemolysin obtained by Yamamoto et al. [10] was a protein with M_r 60000 and p*I* 5.7. It should be noted that for the latter toxin from non-01 *V. cholerae* an amino acid analysis was done, which showed its identity with El Tor hemolysin [19]. The amino acid composition of the cytolysin purified by us was the following: Asp, 65; Thr, 25; Ser, 30; Glu, 61; Pro, 24; Gly, 40; Ala, 48; Val, 31; Met, 2; Ile, 21; Leu, 42; Tyr, 1; Phe, 18; Lys, 17; His, 11; Arg, 18. The analysis indicated that the CC was related, but not identical, to the hemolysin obtained earlier from both El Tor and non-01 strains of *V. cholerae*.

Thus the physicochemical and biological properties of CC allowed us to put the toxin in the group of the thermolabile lethal hemolytic enterotoxins, which are secreted by both classical (El Tor) and non-01 strains of *V. cholerae*. The data presented in our paper allowed us to suggest that a formation in target membranes oligomeric waterfilled channels is the primary mode of action of the studied non-01 *V. cholerae* cytolysin at least.

Properties of the waterfilled channel induced by the CC

The appearance of the current steps after addition of the CC into bathing BLM solution is a strong indication for the formation by the toxin of ionic channels. The data presented above allowed us to affirm that the same ion-conductive structures were formed by the CC into erythrocytes membranes.

The possibility to form an asymmetric lipid bilayer was shown by Montal nearly twenty years ago [29]. The investigations using asymmetric bilayers had been performed, for example, on the channels induced by gramicidin [20], α-staphylotoxin [31]. The asymmetric bilayers used to study the membrane effects of CC allowed us not only to establish the channel-forming activity of the toxin, but also to find the asymmetric location of the CC channels in the membranes. The proteolysis of the definitive CC-channel structure by papain had supported the assumption of the asymmetric location of the CC-channel in BLM. It is obvious that the data obtained do not provide direct evidence for the asymmetric location of the CC channel, but electron microscopy studies have not been done yet.

The simple relation between solution conductivity and conductance of the CC channels and the high permeability of the channels for small non-electrolytes are a strong indication that the CC channel is filled with water, i.e., general diffusion pore. However, the instantaneous CVC of the membrane modified by CC was nonlinear and asymmetric (Fig. 4). CVCs were found to be always hypolinear at positive and hyperlinear at negative potential at any used membrane composition (Table I). These findings imply that the CC channel possesses some charge. The anion selectivity of this channel indicates that these fixed charges are positive. It was supported by the CC-channel pH dependence of both the selectivity and single-channel conductance (Fig. 7). The expected increase in the electrostatic attraction of the anions at low pH manifested as a strong increase of the single-channel conductance. And in concordance with the decrease the positive charge of the toxic protein at alkaline pH values of the medium the cation-anion selectivity of the CC channels was slightly reduced in this pH region. A similar influence of the pH of the media was also shown for channels induced by, for example: hemocyanin [32], α-staphylotoxin [33], latrotoxin [34].

pH-dependent voltage gating of CC-channels

The pH of the medium also influenced the appearance of voltage gating of the CC channels. At small values (< 30 mV) of transmembrane potential (V_m) the CC channels were in the open state. The increase in V_m raised the probability of closed state of the channels. Moreover, the process of transition of these channels from open to closed state sharply depends on bulk solution pH. So the conductance of the multichannel membrane modified by the CC after a stepwise increase in the membrane potential (from −5 to −100 mV) decreased about e times for 200–300 s at pH 7.5 and for 8–12 s at pH 5.0. Back transition of the CC channels from closed to open state could be approached by membrane depolarization for a short time. It is interesting that current amplitude after a series of identical voltage pulses (from −100 to −5 mV) depended on pulse number only but didn't depend on a time interval between the neighbouring depolarizating

pulses. These results allowed us to suggest that the CC-modified bilayer possesses a so-called 'memory' like α-staphylotoxin-modified BLM [35]. The results of a detailed investigation of these properties of the CC channels will be published separately. In conclusion, the CC-channel transitions between open and closed states are dependent on transmembrane potential and are regulated by pH of media.

pH influence on CC-channel formation

To study the pH dependence of the channel formation and function we observed an interesting effect: both the maximum pore formation rate and the steady-state number of channels had a maximum at pH 6.0–6.5 (Fig. 5).

The same pH effects were observed using some other channel-forming proteins [36]. In all cases the pH changed the ion channel distribution between closed and open states. A possible physicochemical explanation of the phenomena has been discussed earlier [36]. It was based on the similar bell-shape pH-dependence of both the Gibbs free energy of interaction of ionogenic groups of a channel-forming protein with water and the total energy of the electrostatic interactions between neighbouring ionogenic groups of channel-forming protein molecules in channel structures.

We have supposed that slight decreases in pH value of the solution bathing the target cells should result in increases in sensibility of the cells to the CC action. This proposition was supported by the results of simple experiments. We have measured leakage of K^+ ions from human erythrocytes treated by the CC at various pH values of the medium. The results are presented in Fig. 8 as the rate of K^+ leakage in 5 min after addition of the toxin to the suspension of human erythrocytes. It can be seen that the rate of ion leakage induced by the CC had a maximum at pH 6.5–7.0. So these data undoubtedly indicate that a slight decrease in pH readily increased the sensibility of the cells to the CC action. The presence of the pH-optimum for membrane effects of the CC and localisation of the optimum (6.0–7.0) on the pH scale allows us to suggest that a combination of such CC properties as both a relative low-discrimination of cytotoxicity to various mammalian cells and a suitable pH-dependence of its poreforming activity will favour the use of this toxin as a chemotherapeutical antitumour medicine. This hypothesis should be tested in vivo, but now it is supported by these facts that the pH value of the extracellular medium bathing the tumour cells could be 0.4–1.0 pH unit lower than that of the interstitial fluid of normal tissues [37].

In conclusion, we should note that the primary mode of, at least, erythrocyte damage by the CC secreted by non-01 *V. cholerae* is the formation water-filled pores in the target membranes.

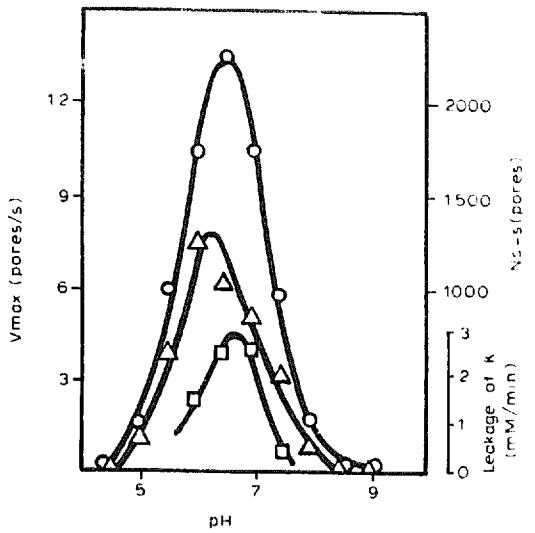

Fig. 8. pH-dependence of the maximum pore formation rate, the steady-state number of CC channels and rate of leakage of K^+ from CC-treated erythrocytes. Bathing solutions containing 150 mM NaCl, 5 mM Tris-OH were adjusted to required pH by citrate. The toxin (final concentration 1.25 μg/ml) was added to cis side of BLM clamped at −20 mV. The values of the maximum pore formation rate (○) and the steady-state number of channels (△) were calculated as described in the legend to Fig. 5. Rate of leakage of K^+ from 2% suspension of human erythrocytes (□) in 5 min after addition of CC to the a final concentration 0.25 μg/ml was calculated as described in Materials and Methods. There were carried out from 2 to 7 experiments to obtain each value of dependences. The lines were drawn by hand.

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