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The ionic channels formed by cholera toxin in planar bilayer lipid membranes are entirely attributable to its B-subunit

Oleg V. Krasilnikov [1], Javdat N. Muratkhodjaev [1], Sergei E. Voronov [2] and Yuri V. Yezepchuk [2]

[1] *Institute of Physiology, Uzbek Academy of Sciences, Tashkent (U.S.S.R.) and* [2] *Gamaleja Institute of Microbiology and Epidemiology, Moscow (U.S.S.R.)*

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The interaction of cholera toxin with planar bilayer lipid membranes (BLM) at low pH results in the formation of ionic channels, the conductance of which can be directly measured in voltage-clamp experiments. It is found that the B-subunit of cholera toxin (CT-B) also is able to induce ionic channels in BLM whereas the A-subunit is not able to do it. The increase of pH inhibited the channel-forming activity of CT-B. The investigation of pH-dependences of both the conductance and the cation-anion selectivity of the CT-B channel allowed us to suggest that the water pore of this channel is confined to the B-subunit of cholera toxin. The effective diameter of the CT-B channels water pores was directly measured in BLM and is equal to 2.1 ± 0.2 nm. The channels formed by whole toxin and its B-subunit exhibit voltage-dependent activity. We believe these channels are relevant to the mode of action of cholera toxin and especially to the endosomal pathway of the A-subunit into cells.

Introduction

The property of cholera toxin (CT) to induce ionic channels into planar lipid bilayers was established by Tosteson and Tosteson more than ten years ago [1]. However, neither the structure of these ionic channels nor their role in the mechanisms of toxins action has been known. It will become clear if, firstly, the contribution of cholera toxin subunits (B and A) and toxin receptor-ganglioside G_{M1} to the cause of ionic channel fenomena is determined. Secondly, the dependence of both the channel-forming activity and the ionic channel properties on the pH of the water solution should be estimated. Lastly, it is necessary to determine if this channel is large enough to allow the A-subunit to pass through it. To study the membrane effects of CT and especially its subunits (CT-A, CT-B) we have used planar bilayer lipid membranes.

Materials and Methods

Planar bilayer lipid membranes (BLM) were formed at room temperature by the union of two monolayers [2] by using a mixture of the phosphatidylserine (PS), phosphatidylcholine (PC) and cholesterol (Chol) (1 : 1 : 1, w/w). Monolayers from a 1 mg/ml solution of this mixture in *n*-hexane were spread and after evaporation of the solvent membranes were formed on a hole in a 20 μm thick Teflon partition separating two buffered salt solutions. The hole (0.2–0.4-mm diameter) was pretreated with a 1 : 20 solution of hexadecane in *n*-hexane. To estimate the radius of CT-B channel we have used the method worked out at our laboratory [3,4]. It is based on the determination of the changes of a single ionic channel conductance by different nonelectrolytes added to the aqueous solution. Bathing solutions, 2 ml on each side, contained 100 mM KCl, 3 mM citrate and were adjusted to needed pH with Tris-OH. In the channel-sizing experiments solutions also contained nonelectrolytes added to a final concentration of 20% (w/v). Glycerine, glucose, sucrose and

Abbreviations: CT-B, cholera toxin B-subunit; G_{M1}, galactosyl-*N*-acetylgalactosaminyl(*N*-acetylneuraminyl)galactosylglycosylceramide; BLM, bilayer lipid membranes; Tris, 2-amino-2-hydroxymethylpropane-1,3-diol; PC, phosphatidylcholine; PS, phosphatidylserine; Chol, cholesterol; PEG, poly(ethylene glycol).

Correspondence: O.V. Krasilnikov, Institute of Physiology, Academy of Sciences, Nijazova 1, Tashkent, U.S.S.R.

poly(ethylene glycol)s (PEG) with average molecular mass (Da): 400 (Schuchardt, F.R.G.); 1000, 1500, 2000, 4000 (Loba Chemie, Austria) were used. Hydrodynamic radii of nonelectrolytes were measured in our laboratory [4] using a viscosimetrical method. The conductivity of each buffer solution was measured with a Radelkis OK 102/1 conductometer.

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, is defined as $G = I/V$, where I is the current flowing through the membrane and V is the potential of the cis compartment. The trans compartment was connected to the virtual ground and voltage signs are referred to it. The cation transference number, t + was calculated by using zero-current potential, E_m observed in the presence of KCl concentration gradient through a membrane according to $t_+ = (E_m - E_a)/(E_c - E_a)$, where E_c and E_a are the respective theoretical Nernst potentials for cations and anions in this system.

The cholera toxin was prepared from culture filtrate of *Vibrio cholerae* O1 by one of us (Voronov, S.) according to Ref. 5. The cholera toxin subunits were separated by gel filtration as described [6]. It is necessary to note that before lyophilization, separated subunits underwent so called 'renaturation' [6]. Such renaturated subunits were obtained by dissolution of these in buffer containing 6 M urea, 100 mM KCl, 3 mM citrate-Tris (pH 3.5), followed by gradual removal of the urea by dialysis. Then the samples were lyophilized and stored at −20 °C.

Usually after the membrane was completely formed and stabilized, a portion of a freshly diluted solution of CT or the desired subunits was added to the cis compartment, to give concentrations ranging from 5 ng/ml to 50 μg/ml. In separate experiments these proteins were added by application of stock solutions on the membrane.

Chromatographically pure phosphatidylcholine (PC) from egg yolk and phosphatidylserine (PS) from ox brain were prepared according to Ref. 7. Cholesterol was purchased from Sigma Laboratories (Munich, F.R.G.).

Results and Discussion

In agreement with results [1] we established that cholera toxin was not able to form channels into BLM, lacked of ganglioside G_{M1} at neutral pH. However, the decrease of pH value allowed us to register single channels induced by CT into BLM containing anionic phospholipid PS, binding cholerogen well too [8]. The value of these channel conductances was close to 55 pS in 100 mM KCl, 3 mM citrate-Tris, pH 4.5 solution

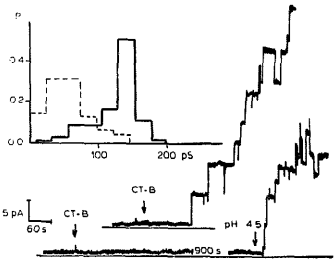

Fig. 1. Current steps through a planar bilayer membranes after the addition of CT-B to the cis compartment. Experimental conditions were: 100 mM KCl, 3 mM citrate-Tris, pH of bathing solution was in the upper trace 4.5, in lower 7.5. Current and time scales are given in the figure, the applied potential was +50 mV, the concentration of CT-B was 12.5 μg/ml. Discrete steps can be observed which each represent the opening of a new ionic channel. In the lower trace the arrow after the interval (15 min) points out the decrease of the pH of solution from 7.5 to 4.5 by citrate resulting in tremendous increase of membrane conductance. The horizontal thin line indicates current zero. (Inset) The histograms representing the probability P to observe steps of CT and CT-B channel conductance like that in the traces. The left histogram (dashed line) shows the probability of whole toxin channel conductance; 48 steps, bin width 24 pS. 5–15 μl of CT was applied from a stock solution (0.5 mg/ml) to the cis compartment of the experimental cell. The right histogram (the CT-B channels); 137 steps, bin width 24 pS, the final concentration of CT-B was 5–10 μg/ml. In common: clamp voltage +50 mV, bathing solution 100 mM KCl, 3 mM citrate-Tris (pH 4.5).

(Fig. 1, inset). The cation transference number, t_+, measured under the same condition was equal to 0.27 ± 0.02, i.e. at low pH the channels formed by CT are anion-selective channels. Its instantaneous current-voltage characteristic was practically linear (Fig. 4). The differences between these properties of CT channels obtained by us and those earlier, established [1] ($G = 20$–40 pS, $t_+ = 0.67$ in 100 mM NaCl (pH 7.0), BLM – from a mixture of a glycerol monooleate and ganglioside G_{M1}) are, perhaps, the result of using different experimental conditions. The fact that cholera toxin was able to induce ionic channels in phosphatidylserine-containing BLM is a strong indication to the presence of ganglioside G_{M1} in the membrane is not obligatory to produces ion-conductive structure.

With the aim to establish the contribution of toxin subunits (CT-A and CT-B) to the production ionic channels by CT we have tested the pore-formation ability of CT-A and CT-B in media with various pH values. It was obtained, that CT-A had no effect on the membrane conductance at all of the tested pH values of the medium even in a final concentration of 0.1 mg/ml. On the contrary, the addition of CT-B to one BLM side resulted in a step-like increase of membrane

conductance (Fig. 1). So only one subunit (CT-B) of cholera toxin possesses single-channel activity under identical experimental conditions.

At low pH (4.5) CT-B formed an ionic channel well, leading to a rapid rise of macroscopic membrane conductance. Increasing the pH of the medium reduces the CT-B activity, and at pH 7.5 the membrane conductance did not change although B-subunit in a high concentration was placed into the BLM bathing solution, but if the pH was subsequently lowered to 4.5 full activity restored (Fig. 1).

Such pH influence is known for many other toxins such as diphtheria [9–11], botulinum [12], tetanus [13,14], membranoactive colicins [15] etc. In common it can be concluded that these toxins obviously gained entry into the target cell via an endosomal pathway.

It is necessary to note that after addition of B-subunit into the bathing solution an increase of the membrane conductance was step by step. It indicates that CT-B really forms a discrete ion-conductive pathway through planar lipid bilayers. The current steps were rather homogeneous in size, implying that each ionic channel carrier the same amount of current. The conductance of such channels was about 125 pS (100 mM KCl, pH 4.5). Under this conditions CT-B channels exhibited anion selectivity ($t_+ = 0.2 \pm 0.04$). The difference in conductance values between these channels and those formed by whole toxin may be a result of A-subunit interference. In spite of this discrepancy we can conclude that the channels formed by CT are entirely attributable to the B-subunit. The influence of pH on the channel-forming activity of CT and its B-subunit, perhaps, is a result of the pH-induced conformational transition of CT that was found [16,17] by fluorescence measurements. The conformational change resulting in exposure of the hydrophobic surfaces of CT-B facilitates the relationship between CT (CT-B) and membranes.

Our primary interest in the CT-B pore was to determine if it was large enough to allow the A-subunit to pass through. It was established (Fig. 2) that the addition of small hydrodynamic radii nonelectrolytes into the BLM bathing solution led to the decrease of CT-B channel conductance. The decrease was proportional to the change of the solution conductivity. This finding indicates that these channels are waterfilled pores because movements of permeant ions and nonelectrolytes through them were similar to these in the bulk solution. After the addition of PEG 1500-PEG 4000 into the BLM bathing solution the single-channels conductance was like the conductance of channels in the initial solution without nonelectrolytes. Therefore those PEG molecule sizes are greater than the CT-B channel water pore size and such large molecules can not pass through the channel. Using a series of nonelectrolytes with different size of molecules we established that the

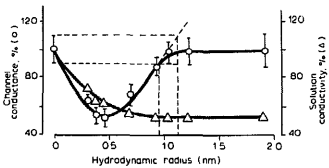

Fig. 2. The effect of nonelectrolytes on the solution conductivity and CT-B channel conductance. Solutions contained 100 mM KCl, 20% (w/v) nonelectrolyte, 3 mM citrate (pH 3.5). Obtained values of channel conductance in these solutions are presented as a percentage of the value of CT-B channel conductance without nonelectrolytes; left vertical scale. Solution conductivity values measured by a conductometer are presented in the same manner. Vertical dashed lines restrict the field taken for a radius of CT-B channels water pore. Nonelectrolytes with the following radii (nm) were used: glycerine, 0.31 ± 0.01; glucose, 0.37 ± 0.02; sucrose, 0.47 ± 0.01; 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.

minimal weight of impermeant PEG molecules was 1500 Da. The hydrodynamic radius of such PEG molecules is equal to 1.05 ± 0.01 nm. Thus the effective diameter of water pores of CT-B channels is about 2.1 nm. This date is in good agreement with the value obtained from electron microscopic studies [18,19] of whole cholera toxin and its B-subunit into membranes. So we can conclude that the ion-conductive channel was exactly an interprotein waterfilled pore constructed by 5–6 B-subunits of CT but not by a ganglioside cluster in micellar-like structures [20].

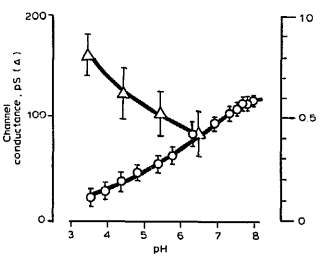

Fig. 3. The effect of pH on the cation-anion selectivity and conductance of CT-B channels. Conductance values, were obtained from incorporated traces like those shown in Fig. 1; left vertical scale. Bathing solutions were 100 mM KCl. The values of cation transport number, t_+, were calculated from a zero-current potential at 3-fold KCl concentration gradient through membrane: trans compartment 40 mM, cis 120 mM. The pH of solution containing CT-B was increased from pH 3.5 by consecutive addition of 0.5 M Tris-OH. After each addition a zero-current potential was measured. The toxin was added in final concentration about 12.5 μg/ml. Other conditions of experiments as described in Materials and Methods.

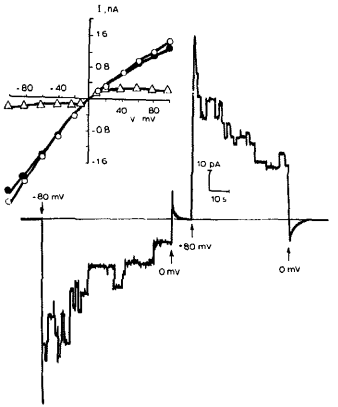

Fig. 4. Voltage dependence of CT-B channels. There are current responses of modified by CT-B bilayers to the changes of applied potential which are indicated by arrows. Current and time scale are given in figure, the horizontal thin line indicates current zero. A final concentration of CT-B was 15 μg/ml. (Inset) Instantaneous (○) and steady-state (△) current-voltage curves were obtained on the membrane containing many CT-B channels. ●, Instantaneous CVC of the membrane modified by whole toxin. Conditions of all experiments were as follows: 100 mM KCl, 3 mM citrate-Tris (pH 4.5). The toxins were added by application of 2–10 μl from a stock solution (0.5 mg/ml) to this cis side of BLM.

For additional support of this conclusion we have investigated the properties of a single CT-B channel in media with various pH values. It was established that either conductance or selectivity of this channel depended on pH the value of the bulk solution (Fig. 3). The channel conductance increased from 85 till 160 pS when the pH of solution was lowered from 6.5 value to 3.5. Simultaneously, the cation-anion selectivity of CT-B channel also changes. So the cation transference number (t_+) changed from 0.6 at pH 8.0 to 0.1 at pH 3.5. All observed changes of channel properties pointed out that the carboxyl groups of toxic protein and, possible, these of membrane lipids take part in its determination. Mainly, anion selectivity of CT-B channels, placed into negatively charged BLM, supported our conclusion that CT-B channel was really a large waterfilled pore surrounded by protein.

The instantaneous current-voltage characteristic (CVC) of membrane modified by CT-B was close to linear whereas the steady-state CVC was of another shape (Fig. 4). The difference between instantaneous and steady-state CVC was a result of the CT-B channels inactivation by a high voltage. The inactivation was time-dependent and it was observed at application of both signs of voltage (Fig. 4). This effect was reversible because a decrease of applied potential revived an initial conductance of the membrane. The existence of reversibility points out that the voltage inactivation is due to, obviously, the changes of channels ability to conduct ions, i.e., transitions between open and closed states, but is not due to a loss of channel structures of the bilayers. The application of high transmembrane potentials to the BLM modified by whole toxin led to the practically identical result, i.e., to the decrease of membrane current (not shown). Moreover, the instantaneous CVCs of both the membrane containing CT channels and the one modified by CT-B channels were also close to each other (Fig. 4). So, obviously, the potential-dependent properties of cholera toxin channel are attributable to the its B-subunit.

In common, potential dependence is wide spread among protein channels. So the channels induced by α-staphylotoxin [21,22], aerolysin [23], cereolysin [24], porins [25,26] and many other proteins possess voltage gating behaviour. For most of these channels also as for CT and CT-B channels the role of this property in mode of toxin action has not been clarified.

Our experiments show that:

contrary to Wisnieski and Bramhall [27] and Tomasi et al. [28] who pointed out that only CT-A was able to penetrate into a membrane, only CT-B really induces ion-conductive channels into a membrane:

at pH 4.5 the CT-B channel is anionic with a mean conductance of about 125 pS in 100 mM KCl;

the ability of CT-B and CT form ionic channels rised by pH decreasing;

the high value of transmembrane voltage cause inactivation of CT and CT-B channels;

the water pore of the CT-B ionic channel was surrounded by proteins. The effective diameter of the CT-B channel water pore (2.1 ± 0.2 nm) measured by us is in agreement with the three-dimensional structure of CT penetrating a lipid membrane where the A-subunit occupies the center of the B-subunit ring [29]. This electron microscopic study demonstrated that the hole and the A-subunit were of the same size. Although, according to Ohtomo et al. [30], the diameter of the A-subunit in water solution reached 3.0 nm. In our opinion, the real size of the water pore of CT-B ionic channels is large enough to allow the A-subunit to pass through. And it is necessary to demonstrate that the CT-A actually passes through B-subunit channels.

Our finding also supports studies [31–35] depicting that cholera toxin undergoes receptor-mediated endocytosis and exposure to acidic pH.

We believe that the induction of the ionic channels under low pH is an essential step in the mechanism of cholera toxin action.

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References

1 Tosteson, M.T. and Tosteson, D.C. (1978) Nature 275, 142–144.
2 Montal, M. and Mueller, P. (1972) Proc. Natl. Acad. Sci. USA 69, 3561–3566.
3 Krasilnikov, O.V., Sabirov, R.Z., Ternovsky, V.I., Merzliak, P. and Tashmukhamedov, B.A. (1988) Gen. Physiol. Biophys. 7, 467–473.
4 Sabirov, R.Z., Krasilnikov, O.V., Ternovsky, V.I. and Merzliak, P.B. (1991) Biol. Membran. 8, 280–291 (in Russian).
5 Mecalanos, J.J., Collier, R.J. and Romig, W.R. (1978) Infec. Immun. 20, 552–558.
6 Lai, C.-Y., Mendez, E. and Chang, D. (1976) J. Infect. Dis. 133 S, S23–S30.
7 Bergelson, L.D., Dyatlovitskya, E.A., Molotkovsky, J.G., Batrakov, L.L. and Prokazova, N.V. (1981) Preparative Biochemistry of Lipids, pp. 66–82, Nauka, Moscow (in Russian).
8 De Wolf, M., Kohn, L.D., Depauw, H., Van Dessel, G., Hilderson, H.J., Lagrou, A. and Dierick, W. (1982) Arch. Int. Physiol. Biochim. 90, B110–B113.
9 Deleers, M., Beugnier, N., Falmagne, P., Cabiaux, V. and Ruysschaert, J.M. (1983) FEBS Lett. 160, 82–86.
10 Montecucco, C., Schiavo, G. and Tomasi, M. (1985) Biochem. J. 231, 123–128.
11 Papini, E., Sandona, D., Rappuoli, R. and Montecucco, C. (1988) EMBO J. 7, 3353–3359.
12 Blaustein, R.O., Germann, W.J., Finkelstein, A. and Das Gupta, B.R. (1987) FEBS Lett. 226, 115–120.
13 Boquet, P. and Duflot, E. (1982) Proc. Natl. Acad. Sci. USA 79, 7614–7618.
14 Hoch, D.H., Romero-Mira, M., Ehrlish, B.E., Finkelstein, A., Das Gupta, B.R. and Simpson, L.L. (1985) Proc. Natl. Acad. Sci. USA 82, 1692–1696.
15 Bullock, J.O., Cohen, F.S., Dankert, J.R. and Cramer, W.A. (1983) J. Biol. Chem. 258, 9908–9912.
16 De Wolf, M.J., Van Dessel, G.A., Lagrou, A.R., Hilderson, H.J. and Dierick, W. (1985) Biochim. Biophys. Acta 832, 165–174.
17 De Wolf, M.J., Van Dessel, G.A., Lagrou, A.R., Hilderson, H.J. and Dierick, W. (1987) Biochemistry 26, 3799–3806.
18 Reed, R.A., Mattai, J. and Shipley, G.G. (1987) Biochemistry 26, 824–832.
19 Ludwig, D.S., Ribi, H.O., Schoolnik, G.K. and Kornberg, R.D. (1986) Proc. Natl. Acad. Sci. USA 83, 8585–8588.
20 Tomasi, M., D'Agnolo, G. and Montecucco, C. (1982) Biochim. Biophys. Acta 692, 339–344.
21 Krasilnikov, O.V., Sabirov, R.Z., Ternovsky, V.I. and Tashmukhamedov, B.A. (1986) Biol. Membr. 3, 1049–1056 (in Russian).
22 Menestrina, G. (1986) J. Membr. Biol. 90, 177–190.
23 Wilmsen, H.U., Pattus, F. and Buckly, J.T. (1990) J. Membr. Biol. 115, 71–81.
24 Krasilnikov, O.V., Usmanova, A.M., Sabirov, R.Z., Tashmukhamedov, B.A., Yezepchuk, Yu.V. and Bicaev, A.R. (1985) Biol. Membr. 2, 302–309 (in Russian).
25 Colombini, M. (1989) J. Membr. Biol. 111, 103–111.
26 Ermishkin, L.N. and Mirzabekov, T.A. (1989) Biochim. Biophys. Acta 1081, 161–168.
27 Wisnieski, B.J. and Bramhall, J.S. (1981) Nature 289, 319–321.
28 Tomasi, M. and Montecucco, C. (1981) J. Biol. Chem. 256, 11177–11181.
29 Ribi, H.O., Ludwig, D.S., Mercer, K.L. and Schoolnik, G.K. and Kornberg, R.D. (1988) Science 239, 36–40.
30 Ohtomo, N., Muraoka, T., Tashiro, A., Zinnaka, Y. and Amako, K. (1976) J. Infect. Dis. 133 S., S31–S40.
31 Joseph, K.C., Kim, S.U., Stieber, A. and Gonatas, N.K. (1978) Proc. Natl. Acad. Sci. USA 75, 2815–2819.
32 Hansson, H.-A., Lange, S. and Lonnroth, I. (1984) Acta Pathol. Microbiol. Immunol. Scand. Sect A. 92, 15–21.
33 Tsuru, S., Matsuguchi, M., Watanabe, M., Taniguchi, M. and Zinnaka, Y. (1984) J. Histochem. Cytochem. 32, 1275–1279.
34 Janicot, M., Clot, J.-P. and Desbuquois, B. (1988) Biochem. J. 253, 735–743.
35 Janicot, M. and Desbuquois, B. (1987) Eur. J. Biochem. 163, 433–442.