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Latrotoxin-induced fusion of liposomes with bilayer phospholipid membranes

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Liposomes containing amphotericin B as ionophoric marker were used to investigate the fusion of bilayer phospholipid membranes with liposomes. It was found that latrotoxin isolated from black widow spider venom induced the fusion of liposomes with planar bilayer when liposomes and latrotoxin were administered at opposite sides of the membrane.

Latrotoxin is a protein neurotoxin isolated from the venom of the black widow spider (*Latrodectus mactans tredecimguttatus*). Current ideas on its mode of action are based on data according to which latrotoxin increases the presynaptic membrane permeability for Ca^{2+} [1] and forms ionic channels permeable for this ion in artificial membranes [2,3]. The fusion of synaptic vesicles with presynaptic membrane is due to the influx of Ca^{2+} into the synaptic ending and causes the massive release of neurotransmitter which in turn leads to devastation of the synapse from the neurotransmitter and complete block of synaptic transmission [4]. This suggestion agrees well with a series of observations on synaptosomes [5], neurosecretory cells [1] and preparations of nerve endings [6].

On the other hand, according to some authors [7,8] the release of neurotransmitter can occur in the absence of calcium ions in the extracellular medium. In view of this fact we suggested the possibility of latrotoxin possesing fusogenic properties. We carried out the experiments with the model system liposome-bilayer phospholipid membrane. This system has been studied rather well and is commonly employed now to study membrane fusion [9–13].

Crude venom was eluated by 0.02 M Tris-HCl buffer (pH 8.05) from venomous glands of adult female species of the black widow spider. This venom solution was frozen and stored at $-70\,^{\circ}C$. The solution was thawed directly before isolation of latrotoxin. Toxin was isolated by means of FPLC liquid chromatography (Pharmacia) with a 'Mono-Q' column which was eluted with a NaCl concentration gradient containing above mentioned buffer. Latrotoxin peak was eluated with 0.33 M NaCl. By polyacrylamide gel electrophoresis and immunoelectrophoresis after rechromatography a homogeneous protein of 130 kDa was obtained. Its LD_{50} was 20 mg/g of mice body weight. This value approaches that of reported by Grasso [14].

Bilayer lipid membranes were formed by the technique of Mueller et al. [15] across a 0.6 mm diameter hole in a teflon cup placed in a glass cell. The membranes were formed from a mixture of phosphatidylcholine and cholesterol (2 : 1, w/w). Heptane was used as a solvent, the lipid con-

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centration was 20 mg/ml. Membrane formation was observed under reflected light with a low-power microscope. The electrolyte solutions around the membrane contained 100 mM KCl, 0.5 mM EDTA and 10 mM Tris-HCl (pH 7.4). 10^{-9} M of latrotoxin was added to the *cis*-side of the membrane.

Liposomes were formed by sonication of 45 mg of phosphatidylcholine, 5 mg of cholesterol and 0.5 mg of amphotericin B in 1 ml of above mentioned solution. Sonication was carried out in a UZIN-2T ultrasonic desintegrator at 22 kHz frequency during 10 min. The liposomes were added to the membrane surrounding solution at a final concentration of 1 mg of lipid per ml. Phosphatidylcholine was isolated from egg yolk according to Ref. 16. Cholesterol was from Serva.

Two Ag/AgCl electrodes connected with both compartments via 3 M KCl in series with pipettes containing agar in 0.1 M KCl were used to transmit the currents through the system under voltage-clamp conditions. While current-voltage curves were being recorded the velocity of potential change was 0.5 mV/s. The *trans*-side potential was zero. Current versus time plots and current-voltage curves were made on an Endim 620.02 *X-Y* recorder.

All experiments were carried out at room temperature, 20–22° C.

After latrotoxin addition at the *cis*-side compartment the membrane conductance was increased as a result of toxin insertion into the phospholipid bilayer. During perfusion of the membrane *cis*-side by the same toxin-free solution its conductance was gradually stabilized. Fig. 1 shows the current-voltage curves of the membrane after perfusion. It is necessary to point out that after perfusion by toxin-free solution the lipid bilayer conductance became stable due to irreversible incorporation of toxin molecules into bilayer membrane [3]. Next, perfusion of the *cis*-side was performed by a solution containing 10 mM KCl, 0.5 mM EDTA and 10 mM Tris-HCl. In these asymmetric conditions the current amplitude and reversal potential were subject to change. The value of reversal potential in asymmetric conditions (10/100 mM KCl) was 55–58 mV. This value approaches quite closely that predicted by the Nernst equation. From this we may conclude

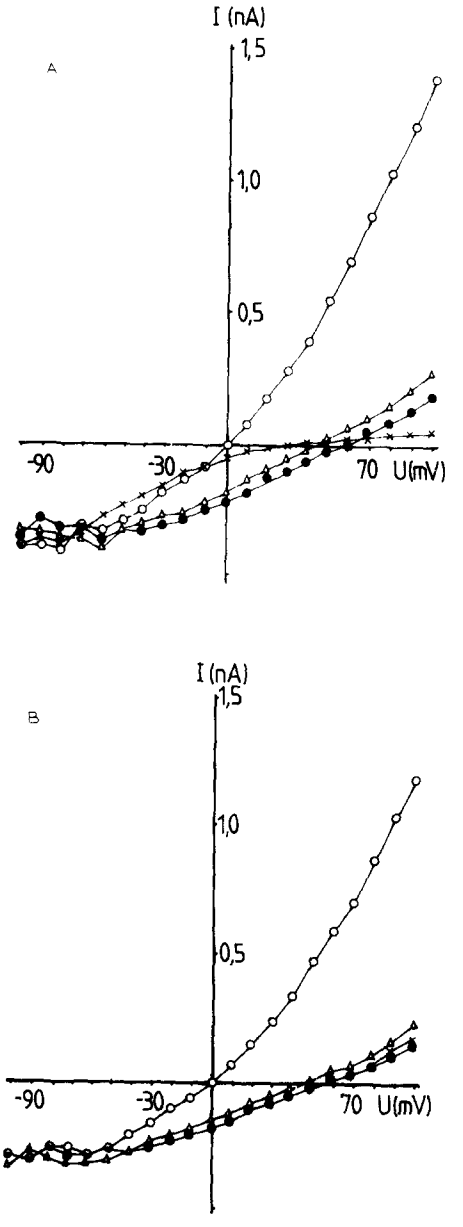

Fig. 1. The influence of amphotericin-containing liposomes on the conductance of bilayer lipid membranes modified by latrotoxin. Current-voltage curves of bilayer lipid membranes modified by latrotoxin: (○) $C^K_{cis} = C^K_{trans} = 100$ mM (A, B); (●) $C^K_{cis} = 10$ mM, $C^K_{trans} = 100$ mM (A, B); (△) after 20 min incubation with amphotericin-containing liposomes at the membrane *trans*-side (A, B); (×) after addition of 2 mM of Cd^{2+} at the membrane *cis*-side in the presence of amphotericin-containing liposomes at the *trans*-side (A); (×) the same after addition of 5 mM of tetraethylammonium at the membrane *cis*-side (B).

that latrotoxin-induced channels possess practically ideal cationic selectivity.

To investigate the interaction between liposomes and bilayer lipid membrane we used amphotericin B incorporated into liposomal membranes as ionophoric marker [9,10]. Comparing the amplitude of single amphotericin channels [18] and latrotoxin channels [3,19] we had to conclude that contribution of amphotericin-induced conductance to the conductance of membrane containing a large number of latrotoxin channels must be negligible. So, we supposed that in this case the changes in the membrane selectivity can indicate its fusion with liposomes. Amphotericin B was chosen because of its predominate anion selectivity [17]. It is known [17] that amphotericin B can form ionic channels in planar bilayer when applied to both sides of it simultaneously. We apply amphotericin-containing liposomes only to one side of bilayers lipid membrane in each experiment. Thus we avoid effects of non-liposome-bound amphotericin B upon the bilayer conductance.

After adding liposomes with amphotericin B at the *cis*-side of membrane we did not observe marked changes in the current amplitude and the reversal potential (not shown). Introduction of the liposomes at the *trans*-side of the membrane caused a gradual increase of current amplitude and a shift of the reversal potential which amounted to 10–15% and 10–12 mV, respectively, at 10–30 min after addition of liposomes (Fig. 1A). Addition of 2 mM of Cd^{2+} which is an effective blocker o latrotoxin channels [3,20], led to significant inhibition of the current amplitude and to a shift of the reversal potential to the anionic permeability side (Fig. 1A). The resulting value of the reversal potential was still cationic but this was explained by incomplete inhibition of the latrotoxin-induced cation permeability.

Completely analogous results were obtained when we inhibited the amphotericin-containing liposome-induced gain of membrane conductance by means of amphotericin B channels blocker tetraethylammonium [21]. As shown in Fig. 1B, administration of 5 mM of tetraethylammonium at the *cis*-side of the membrane causes a decrease of the current and a shift of the reversal potential to values that they had before the liposome administration to the membrane *trans*-side.

Similar results were obtained when we studied the interaction of amphotericin-containing liposomes with the planar bilayer treated by latrotoxin when its channels were blocked by cadmium ions. These results are presented in Fig. 2. It is seen that addition of latrotoxin at the *cis*-side of the membrane was followed by an increase of membrane current. There are distinguished stepwise current jumps in the record. The amplitudes of these steps are corresponding to those obtained for latrotoxin under similar conditions by Robello et al. [19]. Further, while the toxin-free solution was perfused the conductance of the membrane became stable. Addition of Cd^{2+} at the *cis*-side of the membrane decreased the conductance practically to the baseline. *Trans*-side application of amphotericin-containing liposomes causes a slow increase in bilayer membrane conductance. This gain in conductance was not inhibited by Cd^{2+} but addition of tetraethylammonium was accompanied by a decrease in the membrane conductance gain. It is necessary to point out that addition of the liposomes at the *cis*-side of the membrane did not produce any changes in its conductance under similar conditions.

So, we can conclude that the planar bilayer

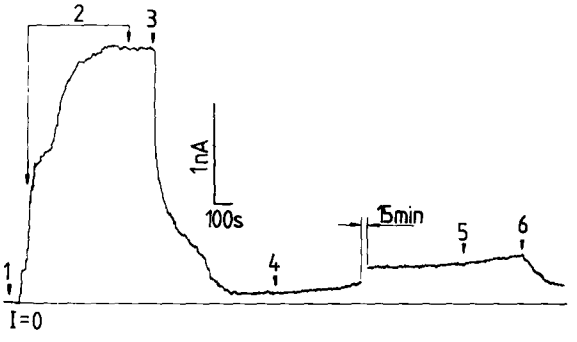

Fig. 2. Interaction of the amphotericin-containing liposomes with bilayer lipid membrane modified by latrotoxin. 1, Administration of 10^{-9} M of latrotoxin at the membrane *cis*-side; 2, perfusion of the *cis*-compartment by toxin-free solution; 3, administration of 5 mM of Cd^{2+} at the membrane *cis*-side; 4, addition of the amphotericin--containing liposomes at the membrane *trans*-side; 5, addition of 5 mM of Cd^{2+} at the membrane *cis*-side; 6, administration of 5 mM of tetraethylammonium at the membrane *cis*-side. The membrane potential was +100 mV. $C^{K}_{cis} = C^{K}_{trans} = 100$ mM.

modified by latrotoxin shows increased conductance and acquires the anionic constituent of the permeability due to the fusion with amphotericin-containing liposomes. The validity of using amphotericin B as ionophoric marker in experiments on fusion study of bilayer lipid membranes with liposomes was shown [9,10], and we carried out all the control experiments described in these papers: (a) applying amphotericin B to the one side of the membrane, (b) administration of amphotericin-containing liposomes to the unmodified membrane and (c) the above mentioned experiments when amphotericin-containing liposomes were added to the *cis*-side of the membrane modified by latrotoxin and did not produce any changes in membrane conductance or in reversal potential. Thus, we can state that observed changes in current amplitude and reversal potential are due to the incorporation of amphotericin B into the bilayer lipid membrane as a result of their interaction with the liposomal membranes. Under our experimental conditions this observation provides the evidence of liposomes fusing with the bilayer membrane, as other ways of amphotericin B insertion into the bilayer membrane were excluded by control experiments. Besides, the absence of effects in control experiments and especially the asymmetry effect of latrotoxin (compare control (c) and Figs. 1A and B) have led us to the conclusion that fusion of liposomes with planar bilayer is due to the latrotoxin inserted into the it.

Recently [3,19] it was shown that latrotoxin molecules incorporate into phospholipid bilayers is an oriented fashion. Experiments with pronase acting from the trans-side of the planar bilayer [19] proved that the latrotoxin channel contains the protein segment protruding into the *trans*-side. These observations can explain the specific effect of 'fusogenic', i.e. protruding, segment of the latrotoxin molecules. According to this suggestion it is valid to consider that the orientation of the latrotoxin in artificial and in presynaptic membrane is similar. In this case the 'fusogenic' segment of the latrotoxin molecule can penetrate into the nerve ending and cause the fusion of synaptic vesicles with presynaptic membrane without the participation of calcium ions in this process.

Based on the data presented in this paper we can say nothing about the mechanisms of fusogenic action of the latrotoxin. We can suggest that latrotoxin incorporated into lipid bilayer can induce local defects in the bilayer structure. As suggested by Chernomordik et al. [22], the stalk mechanism of the trilaminar structure formation supposes that firstly in one of the membrane monolayers a bulging defect is formed and the disruption of the lipid monolayer leads to exposure of the phospholipid hydrocarbon chains to the water phase (Fig. 3a). Then, mutual joining of the bulging defects of monolayers belonging to different membranes (for example, to monolayers of planar bilayer and liposome) leads to a stalk formation between them (Fig. 3b). While the stalk diameter increases the trilaminar structure forms (Fig. 3c). The disruption of the intermediate bilayer leads to the complete membrane fusion (Fig. 3d). A detailed explanation of this process was presented previously [22].

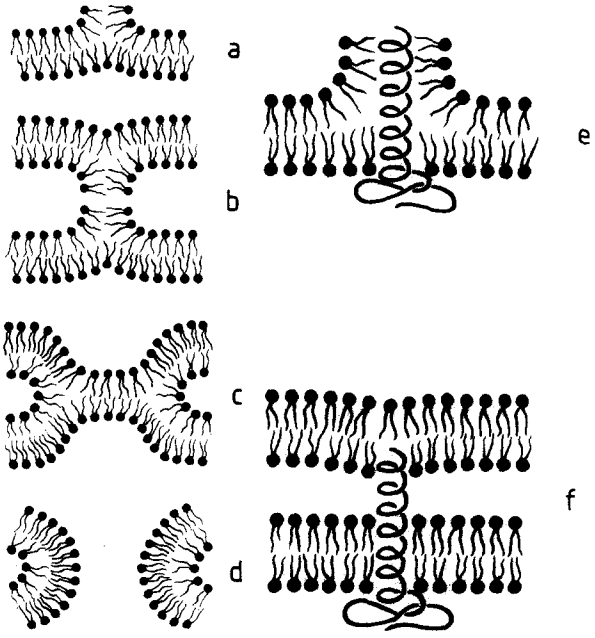

Fig. 3. Scheme of the probable mechanisms of the membrane fusion (a–d) The stalk mechanism [22]. a, Formation of the bulging defects in one of the monolayers; b, a stalk formation between monolayers; c, formation of the trilaminar structure; d, disruption of intermediate monlayer and the fusion; e, formation of the bulging defect as a result of the insertion of a latrotoxin molecule into the lipid bilayer; f, facilitation of the contact between interacting membranes.

These bulging defects formation can occur only in the membranes of definite lipid content [22]. Under our experimental conditions the defects probably did not form (the fusion was absent in the control experiments). One can suggest that also at the places where toxin is incorporated into the membrane bulging defects do occur and resulting, as described above, in disruption of the monolayers. In these places also hydrocarbon chains exposure to water can occur and so, these points can serve as stalk born places (Fig. 3e).

The suggested mechanism of latrotoxin fusogenic action does not exclude other interpretations of this effect. Thus, one can suggest that latrotoxin incorporation does not form the bulging defects and that fusion results from the protruding segment of toxin molecule being inserted into the liposomal membrane (Fig. 3f). It can facilitate the contact between interacting membranes.

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