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DIPHTHERIA TOXIN INDUCES FUSION OF SMALL UNILAMELLAR VESICLES AT LOW pH

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Model membrane systems such as phospholipid vesicles have been extensively used to study the mechanism of membrane fusion at the molecular level. We report here on the capacity of diphtheria toxin to induce fusion of small unilamellar vesicles of dipalmitoylphosphatidylcholine at low pH. Fluorescence polarization and differential scanning calorimetry make it possible to demonstrate the mixing of the lipid phase. Mixing of the internal aqueous compartments of liposome was established using the terbium fluorescence technique. The analogy of structure and properties between melittin and a diphtheria toxin fragment is discussed.

Introduction

Diphtheria toxin consists of a single 62 kDa polypeptide chain. The N-terminal segment (fragment A, $M_r = 22\,000$) inhibits protein synthesis in most mammalian cells by catalytically inactivating elongation factor 2 [1,2]. To interact with elongation factor 2, fragment A must cross a membrane barrier and enter the cytoplasm. The mechanism which has been proposed involves first the binding to a specific surface receptor [1]. In the next step, diphtheria toxin would be endocytosed in acidic vacuoles, probably the endosomes [3]. The low pH inside the endosomes would induce a change in the conformation of diphtheria toxin, exposing an hydrophobic domain which was cryptic at neutral pH. Requirement for an acidic environment has been experimentally demonstrated. Indeed, cells are protected against diphtheria toxin by weak bases like chloroquine and NH_4Cl which accumulate in cell acidic compartments. This protection is removed by exposing cells to a brief pH lowering [4] suggesting that diphtheria toxin is able to directly cross the cytoplasmic membrane. It has been shown that diphtheria toxin [5], CRM 45 (a toxin fragment of 45 000 molecular weight which doesn't contain the carboxyl terminal segment of diphtheria toxin) [6] and CBI (a CNBr peptide of diphtheria toxin B fragment) [7] were able to induce pores formation in black lipid membranes but only at low pH. In this report, we present evidence that diphtheria toxin is able to induce, at low pH, the fusion of small unilamellar lipid vesicles which results probably from a strong destabilization of the lipid bilayer. Melittin is another protein which is capable of inducing fusion of small unilamellar lipid vesicles [8] and forming tetrameric channels in black lipid membranes [9]. Comparison of melittin and a specific region of diphtheria toxin suggests a structural and

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functional analogy between the two proteins although their physiological role are not apparently related.

Materials and Methods

Materials. L-α-Dimyristoylphosphatidylcholine (DMPC) and DL-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Sigma Chemical Company. Diphtheria toxin was a gift from Dr. Falmagne (Université de Mons). 1,6-Diphenyl-1,3,5-hexatriene was obtained from Aldrich. Terbium chloride and dipicolinate were kindly supplied by Dr. Ralston. All reagents and solvents were pro analysi products.

Preparation of vesicles. Phospholipid vesicles were obtained as previously described [10] in a 100 mM Tris-HCl (pH 7.2)/0.9% NaCl buffer or in a acetic acid-acetate (pH 4.2)/0.9% NaCl buffer. Sonication was performed with a Branson sonifier B12 for 15 min. Sonicated suspension was centrifuged at $600 \times g$ for 10 min to remove titanium and residual multilamellar vesicles.

Absorbance measurements. Sonicated dispersions were prepared at a lipid concentration of 0.17 mg/ml. Protein concentration was $2.4 \cdot 10^{-7}$ M. Absorbance was monitored at 550 nm against a liposome blank, using a Shimatzu UV 190 double beam spectrophotometer in 1 cm length cells.

Fluorescence polarization measurements. The fluorescence polarization associated with the hydrophobic probe 1,6-diphenylhexatriene was used to monitor the change in fluidity of the lipid matrix accompanying the gel-liquid crystalline phase transition. The vesicles were labelled by addition of diphenylhexatriene dissolved in tetrahydrofuran (diphenylhexatriene/lipid molar ratio is equal to 1 : 1000). For the experiments performed with a mixture of unilamellar vesicles, sonicated L-α-dimyristoylphosphatidylcholine unilamellar vesicles were maintained in a water bath at 50°C during the sonication of the DL-α-dipalmitoylphosphatidylcholine vesicles. Samples of sonicated vesicles were then immediately mixed in equal quantities. Experiments were carried out with an Elscint microviscosimeter model MV-Ia (Elscint Ltd., Haifa, Israël) designed to give directly the degree of fluorescence polarization P.

$$P = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities polarized parallel and perpendicularly to the direction of polarization of the excitation beam. The heating rate was 2.5 K/min.

Differential scanning calorimetry measurements (DSC). Unilamellar vesicles were prepared as described above at a final lipid concentration of 55 μmol/ml. 90-μl aliquots of lipid suspension and 10 μl of diphtheria toxin (final concentration = 10^{-6} M) or buffer were placed in sealed inox sample pans. A reference sample was similarly prepared using 100 μl buffer. Measurements were carried out on a Setaram DSC III differential scanning calorimeter (Lyon, France) operating at a heating rate of 2 K/min.

Monitoring of liposomes aqueous content mixing. Sonicated unilamellar vesicles were prepared as described above. They were formed either in 10 mM $TbCl_3$/100 mM sodium citrate (pH 7.0) (first population) or in 35 mM sodium dipicolinate (DPA)/Tris-HCl (pH 7.9) (second population) and separated from the non encapsulated material by gel filtration through a Sephadex G-50 column equilibrated with 1 mM Tris/1 mM EDTA (pH 7.2)/0.15 M NaCl buffer. In order to establish the pH dependence of the fusion process, 1 ml of liposomes was brought either to pH 4.2 by addition of 2 ml of 30 mM acetate buffer/1 mM EDTA/0.15 NaCl (pH 4.2) or to pH 7.3 by addition of 2 ml of 1 mM Tris/1 mM EDTA buffer (pH 7.3)/0.15 M NaCl. This pH change was performed immediately before the fluorescence measurement. Diphtheria toxin was added directly into the cuvette and mixed with a pasteur pipette. The final concentration of diphtheria toxin is $1.2 \cdot 10^{-7}$ M. The volume added never exceeded 0.5% (v/v) of the liposome suspension. No sedimentation of the liposomes was observed during the measurement. Lipid concentration in the cuvette was 0.17 mg/ml as determined by the method of Bartlett [11]. Excitation of Tb fluorescence was realized at 276 nm and emission was measured at 491 nm in a Perkin-Elmer model 204 spectrofluorimeter.

Results

Absorbance measurements

The turbidity of dipalmitoylphosphatidylcholine small unilamellar vesicles (SUV) was followed

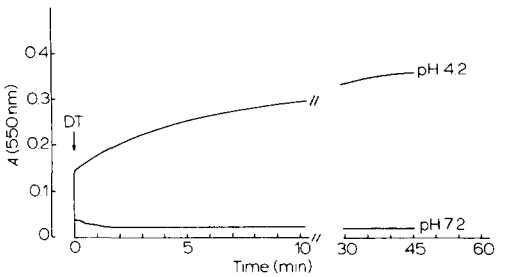

Fig. 1. Turbidity measurements carried out at room temperature on suspensions of small unilamellar vesicles of dipalmitoylphosphatidylcholine. Lipid concentration, $2.3 \cdot 10^{-4}$ M. Protein concentation, $2.4 \cdot 10^{-7}$ M. Protein/lipid molar ratio, 1:958. Time zero corresponds to the addition of diphtheria toxin (DT).

by measuring at room temperature the 550 nm absorbance. These data shown in Fig. 1 indicate that diphtheria toxin is capable of inducing, at low pH, a rapid rise of the turbidity.

Fluorescence polarization

Small sonicated vesicles made of dimyristoylphosphatidylcholine were mixed in equimolar lipid concentration with small sonicated dipalmitoylphosphatidylcholine vesicles. After mixing, and in absence of diphtheria toxin, the polarization curve (Fig. 2A) shows two transitions,

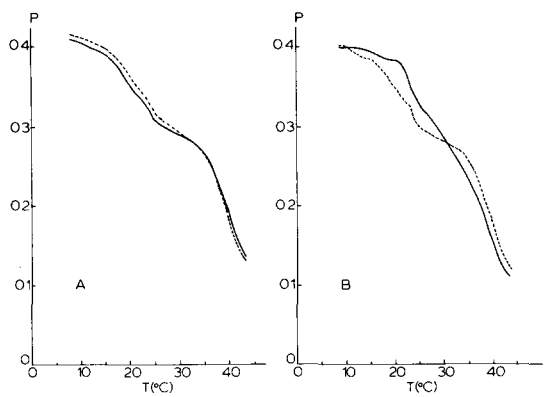

Fig. 2. Fluorescence polarization (P) of diphenylhexatriene solubilized in unilamellar vesicles. Small unilamellar vesicles of dimyritoylphosphatidylcholine (1.1 mol/ml) were mixed in equimolar lipid concentration with small unilamellar vesicles of dipalmitoylphosphatidylcholine. Incubation was performed at 16 °C for 35 min at pH 7.2 (A) and at pH 4.2 (B). - - - - - -, Liposomes without diphtheria toxin; ———, liposomes with diphtheria toxin 10^{-6} M.

characteristic of dimyristoylphosphatidylcholine sonicated unilamellar vesicles and dipalmitoylphosphatidylcholine sonicated unilamellar vesicles. Addition of diphtheria toxin (10^{-6} M) at pH 7.2 doesn't modify the system. At low pH (pH 4.2), we observed a single transition characterizing liposomes made of dimyristoyl- and dipalmitoylphosphatidylcholine in equal amounts (Fig. 2B). The data obtained didn't depend on the incubation temperature (data not shown).

Differential scanning calorimetry (DSC)

Small unilamellar vesicles (SUV) of dipalmitoylphosphatidylcholine were incubated without or with diphtheria toxin, at pH 7.2 and at pH 4.2. Formation of small unilamellar vesicles and multilamellar vesicles were detected by differential scanning calorimetry. Without diphtheria toxin, the DSC pattern shows a main transition temperature (peak a) associated to SUV and a minor transition temperature (peak b) associated to larger vesicles structure resulting from spontaneous destabilization of the vesicles. This system was not modified by adding diphtheria toxin (10^{-6} M) at pH 7.2 (Fig. 3A). On the contrary, at pH 4.2, Fig. 3B shows a significant increase of toxin-induced fusion between small unilamellar vesicles.

Mixing of aqueous phases

The terbium fluorescence method was used in

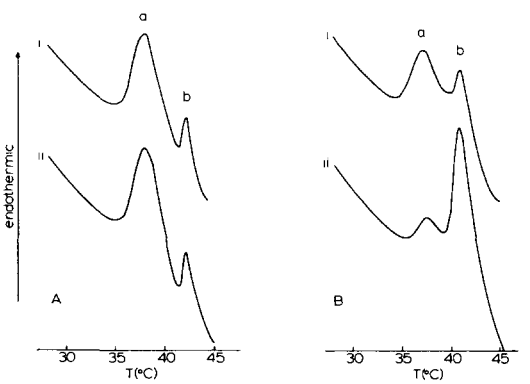

Fig. 3. Differential scanning calorimetry patterns obtained for sonicated vesicles of dipalmitoylphosphatidylcholine (55 μmol/ml) at pH 7.2 (A) and pH 4.2 (B). (i) Liposomes without diphtheria toxin. (ii) Liposomes with diphtheria toxin at a concentration of 10^{-6} M. Diphtheria toxin was incubated for 35 min at 16 °C with the vesicles before scanning.

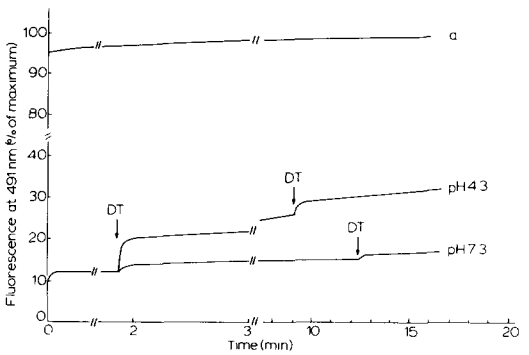

Fig. 4. Terbium fluorescence. After the first addition, diphtheria toxin final concentration is $1.2 \cdot 10^{-7}$ M and $2.4 \cdot 10^{-7}$ M after the second addition. Lipid concentration is 0.17 mg/ml. (a) 100% of fluorescence was obtained by entrapping a mixture of Tb and dipicolinic acid solutions at a 1:1 (v/v) ratio into sonicated vesicles. All the steps of the experiment were performed at room temperature (18 °C).

order to demonstrate that mixing of the internal aqueous compartments of liposomes is associated to the lipid mixing process [8,12]. Liposomes were divided into two populations, one entrapping Tb^{3+} and the other its fluorescence activator dipicolinic acid (DPA). At pH 4.3, mixing of the aqueous compartments was revealed by immediate fluorescence increase after addition of diphtheria toxin to the mixed populations of liposomes (Fig. 4). Doubling the toxin concentration produced only a 17% immediate increase of the fluorescence intensity at pH 4.3, suggesting that the concentration used is nearly optimum for fusion. At pH 7.3, addition of diphtheria toxin induced only a 17% increase of the fluorescence intensity as compared to the fluorescence observed at pH 4.3.

Discussion

Our results indicate that diphtheria toxin induces the fusion of sonicated small unilamellar vesicles at low protein/lipid ratios. Low pH remains a prerequisite to such a process. Absorbance increase observed in presence of diphtheria toxin indicated the formation of larger vesicles but doesn't allow to distinguish between aggregation of these vesicles and real fusion. Fluorescence polarization suggested that diphtheria toxin could induce, at low pH, an exchange or a lipid mixing between two liposomes populations. Moreover, differential scanning calorimetry experiments showed clearly that the interaction between diphtheria toxin and small sonicated vesicles results into the appearance of a new population of vesicles. These vesicles had the thermotropic properties of multilamellar or large unilamellar vesicles. As these vesicles cannot be centrifuged at $600 \times g$, the new population is supposed to the rather made of large unilamellar vesicles than multilamellar vesicles [13]. The mixing of aqueous internal liposomal phase has been demonstrated by Tb^{3+}-dipicolinic acid fluorescence. Comparison of absorbance and Tb^{3+} fluorescence data showed that both phenomenon are displaying a very fast initial increase, followed by a much slower one. At the present time, we have no obvious explanation to account for that bi-phasic profile of the curve. It might be assumed that the Tb^{3+} fluorescence reaches its maximal value as soon as each Tb^{3+}-containing vesicle has fused with a dipicolinic acid-containing vesicle. Subsequent fusion would not improve the mixing of the liposome aqueous content of the two populations of vesicles and would therefore not modify the fluorescence. In absorbance measurements, any supplementary fusion or aggregation could enhance to a certain extent the turbidity of the liposomal suspension and be responsible of a continuous absorbance increase. Total mixing of the liposome aqueous compartments was simulated by entrapping a mixture of Tb^{3+} and dipicolinic acid solutions at a 1:1 (v/v) ratio into sonicated unilamellar vesicles. This ideal system produced a fluorescence increase which was about 7-fold higher than the one produced by diphtheria toxin mediated fusion (Fig. 4). From this result, it can be assumed that the diphtheria toxin mediated fusion process is not total or that leakage of the fluorescent markers or entry of the quencher (EDTA) occurs during the initial step of the fusion. The hypothesis of leakage is difficult to admit since we have recently observed (data not shown) that when diphtheria toxin was added to vesicles entrapping a Tb^{3+}-dipicolinic acid mixture, no fluorescence decrease was observed, suggesting that no leakage and subsequent quenching occurs. When vesicles were disrupted by sodium dodecyl sulphate (0.1% final concentration), the fluorescence decreased dramatically down to 8.7% of the initial value. Our result differs from data

obtained by others with a lytic agent like melittin [8]. In this system, EDTA quenches strongly the fluorescence by entering into the vesicles. Melittin also induces fusion of unilamellar phospholipid vesicles [8] and forms tetrameric channels in lipid bilayers [9]. X-ray data of melittin [14] showed that the protein is made of two α-helixes linked by a proline residue. One of them is amphipathic, shows a large apolar surface (Fig. 5A) and is supposed to insert into the lipid membrane [9]. The predicted secondary structure of diphtheria toxin fragment B [15] revealed a region (residues 134–178) made of two α-helixes linked by a proline residue. Again, one of the helixes is amphipathic with a large apolar surface (Fig. 5B). Furthermore this helical segment has been predicted to be a transverse lipid associating domain [14] and could be made responsible for pore formation in conductance measurements [7]. Transmembrane potential is probably necessary for pore formation in the case of diphtheria toxin, since no significant leakage was observed in our terbium fluorescence experiments. At the present time, we cannot give any explanation for the relationship between the structure and the properties (fusion and pore formation) of the two toxins. However, this analogy of structure between melittin and this peptide of diphtheria toxin B fragment suggests that, like melittin, diphtheria toxin is able to strongly interact with a membrane. The strong destabilization induced by diphtheria toxin might play a role in the translocation of its fragment A. It is generally assumed that diphtheria toxin must pass through the endosome before reaching the cytoplasm. A strong destabilization of the endosome membrane might facilitate the passage of diphtheria toxin A fragment through the lipidic barrier. Fusion would be the consequence of the destabilization of the liposome system. However, we cannot exclude the fact that fusion might play a role by itself in the translocation process. Fusion has indeed been shown to be a crucial step in a related physiological process. It has been demonstrated that the entry of several viruses like Semliki Forest virus [16] into the cells requires virus and endosome membrane fusion induced by viral proteins. Moreover, this fusion seems depend on the low pH of the endosome compartment. Whether or not fusion is involved in the translocation process of diphtheria toxin A fragment requires further investigations.

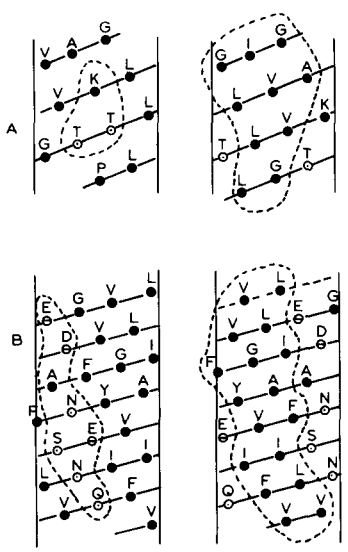

Fig. 5. α-helical nets of melittin (residues 1–14) (Fig. 5A) and of the diphtheria toxin fragment B peptide (residues 153–178) [15] (Fig. 5B). Symbols ●, ⊙, ⊕ and ⊖ represent, respectively, the hydrophobic, neutral, positively and negatively charged residues.

Acknowledgments

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