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Membrane disorganization induced by perfringolysin O (theta-toxin) of *Clostridium perfringens* – effect of toxin binding and self-assembly on liposomes

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θ-Toxin (perfringolysin O) of *Clostridium perfringens* binds to membrane cholesterol with high ($K_d \approx 10^{-9}$ M) and low ($K_d \approx 10^{-7}$ M) affinities and causes membrane lysis of intact cells and liposomes. In order to understand the lytic process at the molecular level, the lysis of liposomes was investigated in comparison with that of intact cells. The toxin dose required to cause 50% lysis (RD_{50}) of phosphatidylcholine/phosphatidylglycerol (82:18, mol/mol) liposomes containing 36–40 mol% cholesterol was 300–1400-times higher than the RD_{50} value for sheep or human erythrocytes when samples with the same cholesterol concentration were compared. However, the average number of toxin molecules bound per liposome vesicle at 50% lysis was estimated as 10–18 from the RD_{50} values, close to the number on erythrocytes at 50% lysis, suggesting that the number of toxin molecules adsorbed per vesicle is important for lysis. As to the toxin dose required for membrane lysis, no significant difference was observed between liposomes containing both high- and low-affinity toxin-binding sites and those containing only low-affinity sites, suggesting that θ-toxin molecules bound to low-affinity sites can assemble and cause membrane lysis as well as those bound to high-affinity sites. θ-Toxin assembles on liposomal membranes, as on erythrocytes, in a high-molecular-weight polymeric form as judged from sedimentation patterns in sucrose density-gradient centrifugation. The high-molecular-weight polymers were detected only under conditions where cell or liposome lysis occurred. At low toxin doses, slower sedimenting toxin oligomers and monomers were predominant on liposomal membranes. These results indicate that toxin assembly on membranes is essential for liposome lysis as it is for cell lysis and that assembly occurs on membranes without membrane proteins.

Introduction

θ-Toxin (perfringolysin O) is an exotoxin produced by *Clostridium perfringens* type A, which belongs to a group of thiol-activated cytolysins [1,2]. These cytolysins bind to membrane cholesterol in a wide variety of mammalian cells resulting in membrane lysis [1–5]. These toxins share extensive structural homologies to one another, especially in their C-terminal regions [6–10]. Studies on isolated C-terminal fragments [11,12] and studies using site-directed mutagenesis [13–15] indicate that the C-terminal portion of cytolysins plays a role in cholesterol binding.

To understand the process of θ-toxin-mediated membrane disorganization, we have prepared θ-toxin derivatives, Cθ and MCθ, which bind specifically to membrane cholesterol without cytolytic effect [16–18]. High- and low-affinity binding sites for θ-toxin ($K_d \approx 10^{-9}$ and 10^{-7}M) exist not only on cell membranes [17,18] but also on liposomes [19,20]. Further analyses on liposomes have revealed that the cholesterol content of liposomes in combination with the phospholipid composition determines the topology of membrane cholesterol as toxin-binding sites [19,20], although it is not yet known whether toxin bound to low-affinity binding sites causes membrane lysis to the same extent as that bound to high-affinity sites.

After binding, the toxin assembles on membranes. Electron microscopic studies reveal that the cytolysins form arc- and ring-shaped structures comprising the assembled forms of several dozen toxin molecules [1,2,21]. The polymeric forms of the toxins can also be detected by sucrose density-gradient analyses. Bhakdi

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Abbreviations: EPC, egg phosphatidylcholine; EPG, phosphatidylglycerol transesterified from EPC; SOPC, 1-stearoyl-2-oleoyl-phosphatidylcholine, POPG, 1-palmitoyl-2-oleoylphosphatidylglycerol.

et al. have shown that the isolated toxin polymers have arc- and ring-shaped structures the same as those observed in electron micrographs [22,23] and suggested that the polymers form membrane-spanning pores through which small and large molecules from the cell interior can pass. However, it has been questioned whether these structures are formed under physiological conditions, since all of the results were obtained in experiments employing 10 000-times the amount of toxin normally required for hemolysis. We have demonstrated by sucrose-density gradient analyses using ^{125}I-θ-toxin that the polymeric forms of θ-toxin are detected under normal hemolytic conditions [11]. Furthermore, we have shown by sucrose-density gradient analysis [11] as well as by electron microscopy [17,18] that the formation of such polymeric structures correlates well with hemolysis.

Although the essential factors for θ-toxin-mediated membrane disorganization, such as toxin binding and assembly on membranes, have been described, the final lytic process has not been evaluated. Rosenqvist and colleagues have reported that streptolysin O, another thiol-activated cytolysin, causes the lysis of liposomes [24], although the low specific activity of their toxin preparations make their results ambiguous. When calculated from their results, the toxin dose required for liposome lysis is more than 100-times higher than the reported dose for hemolysis. For this reason, the possibilities that membrane protein(s) in addition to membrane lipids might play some role in the lytic process or that liposome lysis might occur by a different mechanism from that for hemolysis have not been ruled out. Here we report the results of our investigation on the lysis of liposomes as compared to that of intact cells to better understand whether polymer formation contributes to liposome lysis as in the case of hemolysis and whether membrane protein(s) play a role in the polymer formation. The contribution of high- and low-affinity toxin-binding sites to polymer formation and liposome lysis is also described.

Materials and Methods

Materials

Egg phosphatidylcholine (EPC), phosphatidylglycerol transesterified from EPC (EPG), 1-stearoyl-2-oleoylphosphatidylcholine (SOPC), and 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) were purchased from Avanti Polar Lipids, Pelham, AL. Cholesterol was purchased from Sigma. The purity of the phospholipids and cholesterol was confirmed by thin-layer chromatography as described previously [19,20]. θ-Toxin, Cθ, and ^{125}I-labeled toxins were prepared as described previously [16,17]. One hemolytic unit (HU) was defined as the amount of toxin required to cause 50% hemolysis of sheep erythrocytes (1 ml 0.5% suspension) in 30 min at 37°C.

Preparation of glucose-trapped liposomes

Glucose-trapped liposomes were prepared by removing octyl glucoside from a solubilized lipid sample in the presence of glucose via a slow dilution-dialysis method as described [19,25] with slight modifications. A chloroform solution containing 8.4 μmol of phospholipids (phosphatidylcholine/phosphatidylglycerol = 82:18, mol/mol) and 4.5–5.4 μmol of cholesterol was mixed with octyl glucoside to give a detergent to lipid molar ratio of 10:1; the mixture was then dried on a rotary evaporator. An aqueous solution of 25 mM octyl glucoside in 10 mM Hepes-Na (pH 7.0) containing 0.31 M glucose (buffer A) was added to the dried lipid-detergent sample with vigorous vortexing to give a final lipid concentration of 30 mM. The suspension was slowly diluted 10-fold with buffer A at a rate of 0.3 ml/h under an N_2 stream. The lipid-detergent suspension was then dialyzed against two 1 l changes of buffer A for 2–4 h to form liposomes. An aliquot (3 ml) of the liposome suspension was applied to a 30 ml P6-DG (Bio-Rad) column equilibrated with Hepes-buffered saline (10 mM Hepes-Na (pH 7.0)/150 mM NaCl) to remove residual octyl glucoside and free glucose. The pass-through fractions were collected and used for further analyses within 1 day. The concentrations of cholesterol and phospholipids in the liposome preparations were determined as previously described [19].

Electron microscopic analyses showed size variations of 75–120 nm for SOPC/POPG/cholesterol and EPC/EPG/36 mol% cholesterol liposomes and 60–100 nm for EPC/EPG/40 mol% cholesterol liposomes and that most of the liposomes were di- or trilamellar structures.

The number of vesicles in a suspension was calculated from the lipid concentration assuming 1-to-1 mixtures of di- and trilamellar vesicles with diameters of 100 nm (based on electron microscopic data) and also assuming a bilayer thickness of 4 nm [26] and surface areas for phospholipid and cholesterol molecules of 0.62 nm^2 [27] and 0.39 nm^2 [27], respectively.

Assay for glucose release from liposomes

θ-Toxin was diluted appropriately in phosphate-buffered saline containing 1 mg/ml bovine serum albumin. Glucose-trapped liposomes were diluted in Hepes-buffered saline to give 0.12 mg/ml cholesterol, and 50 μl aliquots of the suspensions were mixed with equal volumes of toxin sample. The mixtures were incubated for 10 min at 37°C, and glucose released from the liposomes was determined after incubation of the mixtures with 0.9 ml of each glucose assay mixture (glucose C-test, Wako Pure Chemical Industries) for 5 min at 37°C. Glucose was oxidized by glucose oxidase

and the hydrogen peroxide produced was quantified by measuring the absorbance at 505 nm after coupling with 4-aminoantipyrine and phenol. The value of 100% glucose release was obtained by parallel assay in the presence of 0.5% Triton X-100.

Hemolysis of erythrocytes by θ-toxin

Erythrocytes were washed three times with phosphate-buffered saline and resuspended in the same buffer. A reaction mixture (0.1 ml) for the hemolysis assay was composed of 50 μl of erythrocyte suspension containing 6 μg cholesterol as a membrane component and 50 μl of θ-toxin solution appropriately diluted in phosphate-buffered saline containing 1 mg/ml bovine serum albumin. After incubation for 15 min at 37°C, hemoglobin release was determined by measuring the absorbance of the supernatant at 540 nm. The number of erythrocytes in the suspension was counted by a hemocytometer after fixing the samples with equal volumes of 2% glutaraldehyde/0.14 M KCl/10 mM sodium phosphate buffer (pH 7.0).

Density gradient analysis of membrane bound θ-toxin

To characterize the features of θ-toxin and Cθ bound to sheep erythrocytes and liposomes, membranes were solubilized with sodium deoxycholate and analyzed on sucrose density gradients as described previously [11]. The binding of ^{125}I-θ-toxin to sheep erythrocytes was analyzed as described [11,17]. The binding of ^{125}I-Cθ to sheep erythrocytes was examined as follows. ^{125}I-Cθ (8.2 ng) was added to 0.5 ml of a 0.3% sheep erythrocyte suspension in phosphate-buffered saline containing 0.8 mg/ml bovine serum albumin. The mixtures were incubated either for 15 min at 37°C or for 60 min at 10°C. After incubation, the erythrocytes were pelleted at 2000 × *g* for 3 min and then hemolyzed with 0.5 ml 5 mM sodium phosphate (pH 8.0). This hemolysis step was omitted for samples incubated at 37°C since the erythrocytes hemolyzed during incubation. After washing the hemolyzed samples, the membranes were suspended in 0.15 ml 5 mM sodium phosphate (pH 8.0) containing 10% (mass/vol.) sodium deoxycholate, and solubilized for 1.5 h at 4°C. Solubilized membrane samples (0.1 ml) were applied to a 3.6 ml linear sucrose gradient (10–50%, mass/vol.) and centrifuged. 15 fractions (0.25 ml each) were collected from the bottom of each tube [11]. The radioactivity was counted, and aliquots were analyzed by SDS/PAGE, autoradiography, and immunoblotting.

For the analysis of liposome-bound θ-toxin, ^{125}I-θ-toxin (5 HU, 4.4 ng) and various amounts of unlabeled θ-toxin were mixed with glucose-trapped liposomes composed of EPC/EPG/40 mol% cholesterol containing 6 μg liposomal cholesterol to make 0.1 ml reaction mixtures in phosphate-buffered saline containing 0.4 mg/ml bovine serum albumin. The mixtures were incubated for 10 min at 37°C and the liposomes were pelleted by centrifugation at 10^5 rpm (Beckman TLA 100 rotor) for 10 min. The liposomes were then washed twice with Hepes-buffered saline, suspended in the same buffer containing 10% sodium deoxycholate, and solubilized for 40 min at room temperature. Solubilized liposome samples (0.1 ml) were analyzed by sucrose-density gradients as described above. The binding of ^{125}I-Cθ to liposomes was analyzed as above except that ^{125}I-Cθ (3.9 ng, 1.3 HU), unlabeled Cθ (500 ng), and SOPC/POPG/39 mol% cholesterol liposomes were used instead of ^{125}I-θ-toxin, unlabeled θ-toxin, and EPC/EPG/40 mol% cholesterol liposomes, and the reaction mixtures were incubated either for 15 min at 37°C or for 60 min at 10°C.

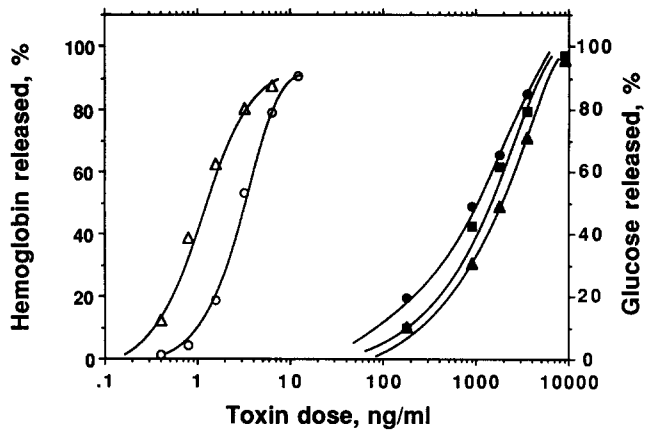

Fig. 1. Dose–lysis plots for θ-toxin on liposomes and erythrocytes. Liposome lysis was measured by the release of trapped glucose (●, ■, ▲) and hemolysis by the release of hemoglobin (○, △) as described in Materials and Methods. Each reaction mixture (0.1 ml) contained 6 μg of liposomal or erythrocyte membrane cholesterol. ●, EPC/EPG/40 mol% cholesterol; ■, SOPC/POPG/37 mol% cholesterol; ▲, EPC/EPG/36 mol% cholesterol liposomes; ○, sheep erythrocytes; △, human erythrocytes.

Results

Comparison of liposome lysis with hemolysis

A series of glucose-trapped liposomes was prepared by removal of octyl glucoside. The lysis of the liposomes by θ-toxin was measured by the release of trapped-glucose and compared with erythrocyte hemolysis (Fig. 1). Toxin dose–lysis plots show a distinctive difference between the toxin doses required for liposome lysis and for hemolysis when samples with the same concentration of membrane cholesterol (60 μg/ml) were compared (Fig. 1). The results shown in Fig. 1 were replotted according to the von Krough equation [28] to give linear plots and the toxin doses required to cause a 50% release of trapped glucose (RD_{50}) from liposomes were estimated. The RD_{50} value for EPC/EPG/40 mol% cholesterol liposomes is 0.85 μg/ml,

TABLE I

Toxin doses for lysis of liposomes and erythrocytes

The θ-toxin doses required to cause 50% lysis (RD_{50}) were obtained by measuring either glucose release from liposomes or hemoglobin release from erythrocytes as described in the legend to Fig. 1. At the RD_{50} concentrations of the toxin the amounts of θ-toxin bound to liposomes or erythrocytes were determined according to the method described previously [17, 19]. Vesicle number was calculated as described in Materials and Methods. PL = phospholipid; Chol = cholesterol.

	Vesicles		RD_{50} (μg/ml)	θ-Toxin bound (%)	θ-Toxin molecules /vesicle
Liposomes	PL	Chol (mol%)			
	EPC/EPG	36	1.75		18
		40	0.85	91	10
	SOPC/POPG	37	1.17	90	12
Erythrocytes					
	Sheep		3.4×10^{-3}	92	25
	Human		1.3×10^{-3}	93	27

250–650-times higher than that required to cause 50% hemolysis of erythrocytes (Table I). When the binding of ^{125}I-θ-toxin to EPC/EPG/40 mol% cholesterol liposomes and to sheep and human erythrocytes was compared at a toxin dose of 44 ng/ml, a concentration at which erythrocytes are lysed but liposomes are not (Fig. 1), more than 90% of the added ^{125}I-θ-toxin bound to liposomes as well as to erythrocytes (data not shown). Therefore the difference in cytolysis between liposomes and erythrocytes cannot be ascribed to the total number of bound toxin molecules. When compared at the RD_{50} concentrations of the toxin, more than 90% of the θ-toxin added was bound to EPC/EPG/40 mol% cholesterol and SOPC/POPG/37 mol% cholesterol liposomes as well as to erythrocytes (Table I). This indicates that the RD_{50} values reflect the total amount of θ-toxin bound to cells and liposomes.

Since the RD_{50} values are much higher for liposomes than for erythrocytes when samples with the same cholesterol concentration were compared, we next examined the average numbers of θ-toxin molecules bound per liposome vesicle or erythrocyte. Since the vesicle sizes of liposomes and erythrocytes are quite different, there is also a considerable difference in the number of vesicles when the samples are normalized for cholesterol concentration. At the toxin dose required for 50% lysis, the average numbers of toxin molecules bound per vesicle were estimated to be 10–18 for liposomes and 21–27 for erythrocytes (Table I). Thus, the number of θ-toxin molecules per vesicle at RD_{50} is quite similar for liposomes and erythrocytes, despite the remarkable difference in toxin dose required for 50% lysis. This suggests that the number of toxin molecules adsorbed per vesicle is important for lysis.

The mode of θ-toxin binding to liposomes depends on the lipid composition of the liposomes [19,20]. EPC/EPG liposomes containing 40 mol% cholesterol and SOPC/POPG liposomes containing 37 mol% cholesterol have both high- and low-affinity toxin-binding sites, while EPC/EPG liposomes containing 36 mol% cholesterol have only low-affinity sites [20]. θ-Toxin releases trapped glucose from EPC/EPG/36 mol% cholesterol liposomes (Fig. 1), although the RD_{50} is slightly higher than for liposomes that contain high-affinity sites (Table I). These results indicate that toxin that binds to low-affinity sites, like that binding to high-affinity sites also causes the lysis of liposomes.

Detection of the assembled form of θ-toxin on liposomes and erythrocyte membranes

Since it appears that the number of toxin molecules bound per vesicle is important for lysis, we next examined toxin assembly on membranes. Based on sucrose-density gradient centrifugation analysis it has been reported that following binding θ-toxin assembles on erythrocyte membranes to form polymers [11,12,22]. The mode of θ-toxin polymer formation on liposomes was compared with that on erythrocytes. Erythrocytes and liposomes were incubated with 4.4 ng of ^{125}I-θ-toxin in 0.1-ml reaction mixtures, resulting in the lysis of erythrocytes but not of liposomes (Fig 1). Following binding, the membranes were pelleted by ultracentrifugation and solubilized in deoxycholate. The distribution of ^{125}I-θ-toxin was analyzed by sucrose-density gradient analysis (Fig. 2). ^{125}I-θ-Toxin bound to erythrocytes was recovered in the fractions near the bottom of the gradient (Fig. 2, closed circles), indicating that the θ-toxin had formed polymers [11]. On the other hand, θ-toxin bound to liposomes shows a different gradient pattern (Fig. 2, open circles). Approximately half of the toxin was recovered at a monomer position (fraction 13). While the other half was recovered in fractions sedimenting more slowly than θ-toxin polymers. Thus, while θ-toxin polymers were detected on lysed erythrocyte membranes [11], such polymers were rarely detected on liposomes under non-lytic conditions. These results are consistent with the estimated number of θ-toxin molecules per erythrocyte of 34, while the number per liposome vesicle is estimated as 0.11.

Dose-dependent assembly of θ-toxin on liposomes

The dose dependency of toxin assembly was examined. EPC/EPG/40 mol% cholesterol liposomes incubated with various amounts of θ-toxin were solubilized and the θ-toxin bound to the liposomes was analyzed on sucrose-density gradients (Fig. 3). When liposomes

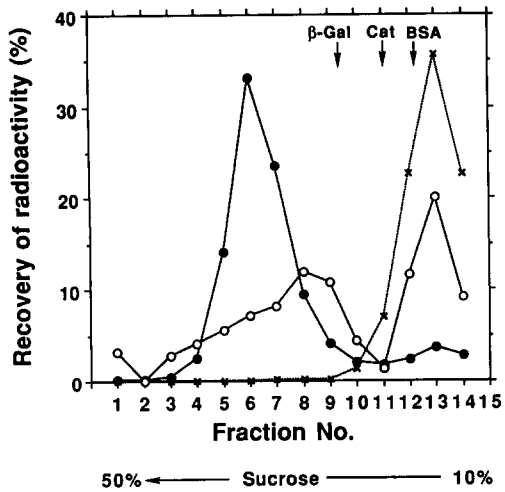

Fig. 2. Comparison of θ-toxin assembly on liposomes with that on erythrocytes by sucrose-density gradient analysis. ^{125}I-θ-Toxin (4.4 ng) was incubated in a 0.1-ml reaction mixture for 10 min at 37°C with sheep erythrocytes (●) or EPC/EPG/40 mol% cholesterol liposomes (○) containing 6 μg membrane cholesterol. Membranes and bound θ-toxin were pelleted by ultracentrifugation and solubilized in 10% sodium deoxycholate. Solubilized samples were analyzed in 10–50% sucrose-density gradients as described in Materials and Methods. ×, free ^{125}I-θ-toxin. Arrows indicate the positions of marker proteins in the gradient. β-Gal, β-galactosidase (16 S); Cat, catalase (11 S); BSA, bovine serum albumin (4.4 S).

were incubated with 160 and 810 ng of θ-toxin (θ-toxin/vesicle ratios = 19 and 93, respectively), 59% and 93% of the glucose was released, respectively (Table II). At these toxin doses θ-toxin solubilized

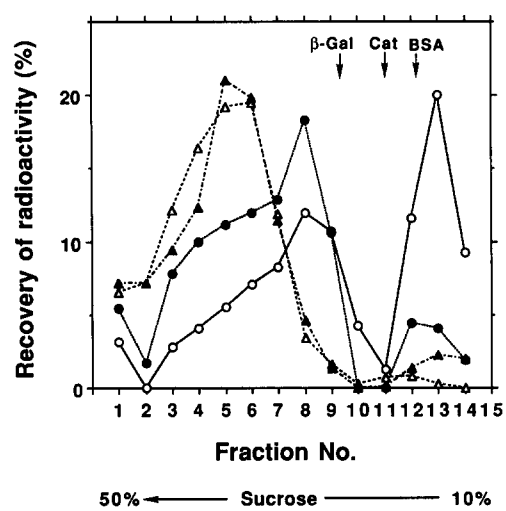

Fig. 3. Dose-dependent θ-toxin assembly on liposomes. ^{125}I-θ-Toxin (4.4 ng) was incubated with EPC/EPG/40 mol% cholesterol liposomes in the absence (○) or presence of 16.2 (●), 160 (△) or 810 (▲) ng of unlabeled θ-toxin in 0.1-ml reaction mixtures for 10 min at 37°C. The assembly patterns of membrane-bound θ-toxin were analyzed using 10–50% sucrose-density gradients. Details are described in Materials and Methods. Arrows indicate the positions of marker proteins (see the legend to Fig. 2).

TABLE II

Toxin-dose dependency of glucose release from liposomes

θ-Toxin and glucose-trapped EPC/EPG/40 mol% cholesterol liposomes were incubated at 37°C and released glucose was assayed as described in Materials and Methods. Data represent mean ± S.E. for three determinations. In parallel experiments, θ-toxin assembly under the same conditions was examined by sucrose-density gradient analysis and the amounts of θ-toxin in high-molecular weight polymers (HMW), slower sedimenting toxin oligomers (oligo), and monomers (mono) were estimated from the density gradient patterns. Vesicle number was calculated as described in Materials and Methods.

θ-Toxin added (ng)	θ-Toxin molecules /vesicle	θ-Toxin (% of total cpm) in			HMW particles [a] /vesicle	Glucose released (%)
		HMW	oligo	mono		
4.4	0.5	15	39	42	0.0027–0.0038	1.1 ± 1.9
21	2.4	37	48	10	0.031 –0.043	11 ± 8
160	19	82	9	2	0.54 –0.76	59 ± 7
810	93	77	10	6	2.6 –3.6	93 ± 6

[a] Assuming that a particle contains 20–28 monomers [21].

from the liposomes was recovered mainly in the fast sedimenting fractions of the sucrose-density gradients (Fig. 3, open and closed triangles). The sedimentation positions are nearly the same as for θ-toxin polymers solubilized from erythrocyte membranes (Fig. 2, closed circles and Ref. 11). On the other hand, when liposomes were incubated with lower amounts of toxin, 4.4 and 21 ng (θ-toxin/vesicle ratios = 0.5 and 2.4, respectively), only 1% and 11%, respectively, of the glucose was released (Table II). The sedimentation patterns in sucrose-density gradients for solubilized θ-toxin from liposomes incubated with high toxin doses were quite different from those of liposomes incubated with low doses (Fig. 3, open and closed circles). In accordance with the decrease in toxin dose, the amounts of toxin recovered at the sedimentation position of θ-toxin polymers decreased and, instead, the toxin appeared in more slowly sedimenting fractions (fractions 8 and 9), suggesting that θ-toxin oligomers with smaller molecular weights than the polymers might have been formed at low toxin doses. The sedimentation coefficient for the oligomers was estimated roughly as 18–22 S. In addition, the relative amount of toxin in the monomer form increased with the decrease in toxin dose (Fig. 3).

Toxin assembly and cytolysis

Next we examined the correlation between cytolytic activity and the degree of toxin assembly. For this purpose we used Cθ, a protease-nicked derivative of θ-toxin. Cθ causes the lysis of cells and liposomes at 37°C, but not at 20°C or lower temperatures even though it binds to membrane cholesterol at these temperatures (Ref 17 and unpublished results). The assembly of ^{125}I-Cθ on liposomes was compared at 10°C and

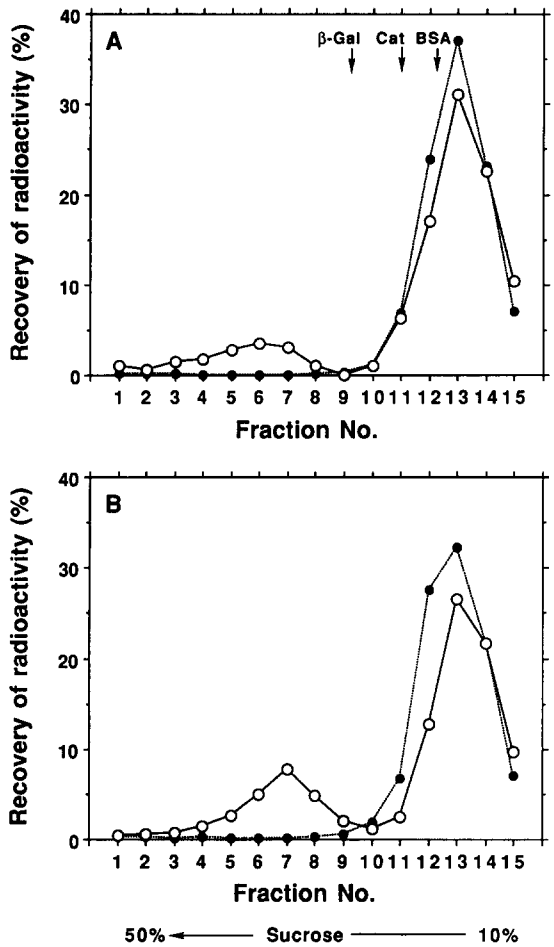

Fig. 4. Assembly of ^{125}I-Cθ on liposomes and erythrocytes at 37°C and 10°C. (A) ^{125}I-Cθ (3.9 ng, 1.3 HU), unlabeled Cθ (500 ng), and SOPC/POPG/39 mol% cholesterol liposomes containing 6 μg membrane cholesterol were mixed and incubated in 0.1-ml reaction mixtures for 15 min at 37°C (○) or for 60 min at 10°C (●). (B) ^{125}I-Cθ (8.2 ng) was mixed with 0.5 ml 0.3% sheep erythrocytes and incubated for 15 min at 37°C (○) or for 60 min at 10°C (●). The assembly patterns of membrane bound Cθ were analyzed using 10–50% sucrose-density gradients. Arrows indicate the positions of marker proteins (see the legend to Fig. 2).

37°C by sucrose-density gradient analysis. A ratio of Cθ molecules to vesicles of 55:1 was used in order to be sure of polymer formation. The polymer form of the toxin was detected only at 37°C, not at 10°C (Fig. 4A), a finding that correlates well with liposome lysis. Although the recovery of radioactivity from Cθ sedimenting in the high-molecular-weight form was smaller than in the case of θ-toxin (Fig. 2), this can be explained by the fact that iodination of Cθ reduces its cytolytic activity to 15–30% of control Cθ without affecting its binding activity. Similar results were obtained from an analysis of the density-gradient patterns of Cθ incubated with erythrocytes (Fig. 4B). These results suggest that the formation of polymers on membranes is essential for liposome lysis as well as for cell lysis.

Discussion

Although the θ-toxin dose required for liposome lysis is 300–1400-times higher than that for hemolysis, the toxin numbers bound per cell or liposome are quite similar at the toxin concentration required for 50% lysis (Table I). These results, along with the finding that the polymer form of the toxin is detected only under conditions where liposome lysis occurs, strongly suggest that θ-toxin assembly on membranes is essential for liposome lysis as it is for hemolysis. It also appears that both θ-toxin assembly and toxin-induced membrane lysis occur in the absence of membrane proteins.

The high-molecular-weight polymers detected on liposomes migrate at a similar position on sucrose-density gradients as the polymers on erythrocytes (Figs. 2 and 3) suggesting that they have similar molecular sizes. We have roughly estimated the S value of the peak polymer fractions (peaks 5 and 6 in Fig. 3) as 26–28 S, corresponding to molecular weights of 900 000–1400 000 for soluble globular proteins. Based on data obtained from electron microscopy it has been reported that ring structures of θ-toxin consist of 20–28 monomers [21], which corresponds to 1100 000–1500 000 as the molecular weight of θ-toxin polymers. From similar analysis, 25–100 monomers have reported to constitute a ring structure of streptolysin O [29], another cholesterol-binding cytolysin belonging to the family of thiol-activated cytolysins. These observations suggest that the toxin polymer detected by sucrose-density gradient analysis may correspond to the ring structure observed by electron microscopy. Assuming that a polymer consists of 20–28 monomers [21], the number of toxin particles bound per vesicle was estimated (Table II). The number of high-molecular-weight particles bound per vesicle correlates well with the degree of liposome lysis.

On the other hand, toxin oligomers with smaller S values (peaks 8 and 9 in Fig. 3) were detected on liposomes when the toxin/vesicle ratio was low. We could not detect this oligomer form on erythrocytes, since in experiments using erythrocytes low toxin/cell ratios are hard to obtain due to the detection limit of the radioactivity of the ^{125}I-θ-toxin used. At low toxin/vesicle ratios, toxin polymers of smaller size would be expected since the number of toxin molecules per vesicle available for assembly must be small. The oligomers detected at low toxin/vesicle ratios might be smaller size, intermediate form of the high-molecular-weight polymers. Although even θ-toxin/vesicle ratios as low as 0.5 a considerable population of ^{125}I-θ-toxin can be detected in the oligomer form (Fig. 3, open circles and Table II), this can be explained by the observation that toxin monomers are exchangeable between membranes, but the assembled form is not [16].

Movable monomers could change the distribution of toxin molecules on vesicles causing the accumulation of oligomers on a few vesicles. So far, we do not know whether the oligomer form of the toxin participates in cytolysis.

It should be noted that similar amounts of toxin molecules per vesicle trigger lysis of liposomes or erythrocytes in spite of the great difference in erythrocytes versus liposomes on the surface density of bound toxin molecules. A θ-toxin molecule bound to erythrocytes, when compared with that bound to liposomes, must diffuse in much larger area of cell surface to associate with other θ-toxin molecules. Since diffusion coefficients of plasma membrane lipids have been reported to be the order of $1 \cdot 10^{-8}$ cm^2/s [30,31], the high diffusibility of membrane cholesterol might bring rapid self-association of θ-toxin molecules bound to cholesterol in membranes.

We have previously demonstrated that two kinds of θ-toxin-binding sites with high and low affinities exist on intact cell membranes [17,18] and liposome membranes [19,20]. The high-affinity sites were detected only on liposomes with high cholesterol contents [20]. The number of high-affinity sites also depends on the chain length and composition of the phospholipids [19,20]. From this observation we have proposed that θ-toxin preferentially binds to cholesterol molecules in cholesterol-rich regions surrounded by phospholipids with 18-carbon hydrocarbon chains [19, 20]. When the lysis of liposomes containing only low-affinity toxin-binding sites (EPC/EPG/36 mol% cholesterol liposomes) was compared with the lysis of liposomes containing both high- and low-affinity sites (EPC/EPG/40 mol% cholesterol and SOPC/POPG/37 mol% cholesterol liposomes), the RD_{50} values were not significantly different (Fig. 1 and Table I), suggesting that toxin bound to low-affinity sites can cause membrane lysis. It should be noted that the maximum number of low-affinity sites on EPC/EPG/ 36 mol% cholesterol liposomes was estimated as approximately 50 per vesicle, which is sufficient for the toxin to form polymers. However, it would be expected that lysis would rarely occur on liposomes that contain far fewer toxin-binding sites per vesicle due to a lack of toxin assembly. Rosenqvist and colleagues have reported that lysis by streptolysin O of large unilamellar liposomes composed of EPC and cholesterol is dependent on cholesterol content and that lysis occurs only with liposomes containing greater than 33 mol% cholesterol [24]. Their results may be explained by the reduced number of toxin-binding sites per vesicle.

There exist many pore-forming bacterial cytolysins [23,32,33] along with thiol-activated cytolysins. Perforins [34,35] and complement protein C9 [36,37] have also been reported to cause membrane damage by self-aggregation. Since it is indicated that liposome lysis occurs by essentially the same mechanism as hemolysis, an analysis of the interaction of liposomes with θ-toxin, in combination with θ-toxin derivatives and fragments which bind to cholesterol but do not cause cytolysis, would provide a good system in which to elucidate the molecular mechanisms of insertion and self-assembly of pore-forming proteins.

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