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Effect of lipidic factors on membrane cholesterol topology – mode of binding of θ-toxin to cholesterol in liposomes

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We have previously suggested the existence of two distinct states for cholesterol in cell membranes as revealed by high- and low-affinity binding sites for θ-toxin of *Clostridium perfringens*. In liposomes, phospholipid and cholesterol compositions, but not membrane protein composition, have been shown to be major determinants for the topology of membrane cholesterol. The effects of lipidic factors on cholesterol topology were investigated in detail by analyzing toxin binding to large unilamellar liposomes composed of cholesterol and phospholipids (neutral phospholipids/phosphatidylglycerol = 82:18, mol/mol). The numbers of high- and low-affinity toxin-binding sites depend strictly on the cholesterol mole percentage in liposomes. High-affinity toxin-binding sites appear only in liposomes with high cholesterol contents. Liposomes whose cholesterol/phospholipid ratio is 0.4 or less have no high-affinity sites regardless of their phospholipid compositions, while low-affinity sites appear in liposomes with lower cholesterol contents. The threshold values for the cholesterol mole percentage above which high-affinity toxin-binding sites appear were examined. The values decrease in accordance with the increase in the mole fraction of 18-carbon hydrocarbon chains among the total 14–18 carbon-hydrocarbon chains of the liposomal phospholipids. Furthermore, both the partial replacement of phosphatidylcholine with phosphatidylethanolamine and the digestion of phospholipids with phospholipase C also affect the threshold values. Thus the cholesterol mole percentage, in combination with phospholipid chain length and other factors, determines the topology of membrane cholesterol providing distinctively different affinity sites for θ-toxin.

Introduction

Cholesterol is one of the major constituents of biological membranes and lipoproteins. It has an important influence on the structure and function of biological membranes, as well as in the pathology of atherosclerosis and cholesterol-storage disorders such as type-C Niemann-Pick disease [1–5]. However, interesting questions concerning its organization and behavior in plasma membranes and intracellular organelles remain to be answered.

Cholesterol functions as a receptor for thiol-activated cytolysins [6] as well as for polyene antibiotics [7] and saponins [8]. θ-Toxin (perfringolysin O) is one of the thiol-activated cytolysins produced by *Clostridium perfringens* [6,9]. This group of toxins binds to membrane cholesterol, thus causing membrane damage in wide variety of mammalian cells [6,10,11]. The thiol-activated cytolysins are suggested to have the same underlying mechanism for membrane lysis because of close similarities in their structures and other characteristics [12–15]. It has been reported that approx. 1.6 molecules of cholesterol neutralize the activity of one molecule of a thiol-activated cytolysin [16], suggesting that one molecule of thiol-activated cytolysin binds one or two molecules of cholesterol.

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Abbreviations: PC, phosphatidylcholine; PG, phosphatidylglycerol; PE, phosphatidylethanolamine; SM, sphingomyelin; EPC, egg PC; EPG, PG transesterified from EPC; EPE, PE transesterified from EPC; MOPC, 1-myristoyl-2-oleoyl-PC; POPC, 1-palmitoyl-2-oleoyl-PC; POPG, 1-palmitoyl-2-oleoyl-PG; SOPC, 1-stearoyl-2-oleoyl-PC; DPPC, dipalmitoyl-PC; DPPG, dipalmitoyl-PG; DSPC, distearoyl-PC; DSPG, distearoyl-PG; C-16, 16-carbon hydrocarbon chains; C-18, 18-carbon hydrocarbon chains.

In order to study the organization of cholesterol in membranes and lipoprotein particles, we have obtained specific probes that bind membrane cholesterol with high affinity but without cytotoxicity [17–20]. Such probes, Cθ and MCθ, were obtained by modification of θ-toxin. Cθ is a protease-nicked derivative of θ-toxin [17], while MCθ is produced by the reductive methylation of Cθ [18]. Cθ and MCθ are useful probes for studying membrane cholesterol because (i) they bind specifically to membrane cholesterol with high affinity but not to other membrane constituents; and (ii) MCθ causes no obvious membrane damage at temperatures of 37°C or lower while Cθ does so only below 20°C [18,19]. Using these probes we have demonstrated previously the existence of two classes of cholesterol as toxin-binding sites, a high-affinity site ($K_d \sim 10^{-9}$ M) and a low-affinity site ($K_d \sim 10^{-7}$ M), in intact cells, such as erythrocytes [18,19] and lymphoma B cells [18], and in liposomes composed of phospholipids and cholesterol [20]. From these findings we proposed the existence of at least two distinctive states for cholesterol in the membranes of these cells and liposomes. Membrane constituents that affect the number and affinity of toxin binding were investigated using liposomes; it was demonstrated that the topology of cholesterol in the membrane is determined by cholesterol–phospholipid and/or cholesterol–cholesterol interactions without the involvement of membrane proteins [20]. The chain length of liposomal phospholipids was also shown to be one of major determining factors for cholesterol topology, at least in liposomes containing 30 mol% or more cholesterol [20]. We report here a detailed analysis of toxin binding to liposomes containing 0–43 mol% cholesterol and various phospholipid compositions to identify membrane components that affect the topology of membrane cholesterol. The cholesterol mole percentage in liposomes is shown to affect toxin binding drastically. An effect on toxin binding of chain length and head group variation in the liposomal phospholipids in combination with cholesterol mole percentage is also demonstrated.

Materials and Methods

Materials. Synthetic phospholipids, phosphatidylcholine (PC) from egg (EPC), phosphatidylglycerol (PG) transesterified from EPC (EPG), and phosphatidylethanolamine (PE) transesterified from EPC (EPE) were purchased from Avanti Polar Lipids, Pelham, AL. *N*-Oleoylsphingomyelin (SM) and cholesterol were purchased from Sigma; 4-cholesten-3-one was from Nacalai Tesque, Kyoto. Purity of the phospholipids and cholesterol was confirmed by thin-layer chromatography as described previously [20]. Cθ, MCθ, ^{125}I-Cθ and ^{125}I-MCθ were prepared as described previously [18,19].

Preparation of large unilamellar liposomes. Large unilamellar liposomes were prepared by removal of octyl glucoside from a solubilized lipid sample using a slow dilution-dialysis method as described in a previous report [20]. Briefly, a mixture of phospholipids, cholesterol (and/or 4-cholesten-3-one), and octyl glucoside (lipid/octyl glucoside = 1:10, mol/mol) was dried on a rotary evaporator and an aqueous solution of Hepes-buffered saline (pH 7.0) containing 25 mM octyl glucoside was added to the dried sample with vigorous vortexing to give a final lipid concentration of 30 mM. The suspension was slowly diluted 10-fold with Hepes-buffered saline under an N_2 stream. Both the lipid-detergent suspension and the diluting buffer were kept at a temperature above the phase transition temperature of the lipid being used. The suspension was then dialyzed against Hepes-buffered saline and the liposomes were collected by ultracentrifugation. The concentration of phospholipids in the liposome preparations was determined by inorganic phosphorus analysis. The concentrations of cholesterol and 4-cholesten-3-one in the liposomes were determined as previously described [19,20].

Cholesterol oxidase susceptibility of liposomes. Liposomes containing 135 nmol of total lipids were incubated with 1 unit of cholesterol oxidase from Nocardia sp. (Oriental Yeast, Tokyo) in 0.5 ml of Hepes-buffered saline either for 3 h at 10°C or for 90 min at 25°C; the oxidation of liposomal cholesterol to 4-cholesten-3-one was measured as described by Moore et al. [21]. As a control for 100% oxidation, an aliquot of each preparation of liposomes was disrupted with 0.05% Nonidet P-40 and incubated with cholesterol oxidase for 30 min at 37°C.

Treatment of liposomes with phospholipase C. Liposomes containing 120 μg/ml cholesterol were incubated with various amounts of phospholipase C (from *Clostridium perfringens*, Sigma type XIV) in Hepes-buffered saline (pH 7.0) containing 0.65 mM Ca^{2+} for 30 min at 25°C. Each reaction mixture (0.6 ml) was divided into two equal aliquots and one aliquot was washed once with Hepes-buffered saline containing 0.5 mM EGTA and then twice with Hepes-buffered saline without EGTA. The cholesterol contents of the washed liposomes were then determined and the binding of Cθ to the liposomes was measured as described [20]. The other half of the reaction mixture (0.3 ml) was mixed with 1.125 ml of chloroform/methanol (1:2, v/v), vortexed, and left to stand for 1 h. Then, 0.375 ml each of chloroform and distilled water were added and the amounts of phosphorylcholine released from phospholipids, recovered in the upper methanol-water phase, were determined by inorganic phosphorus analysis. The percentage of phospholipids hydrolyzed was deter-

mined from the ratio of the amount of inorganic phosphorus in the upper phase to the amount of total inorganic phosphorus.

Results

Effects of the cholesterol mole percentage in 1-stearoyl-2-oleoyl-PC (SOPC) / 1-palmitoyl-2-oleoyl-PG (POPG) / cholesterol liposomes on toxin binding

A series of large unilamellar liposomes composed of SOPC/POPG (82:18, mol/mol) and 0–41 mol% cholesterol were prepared by removal of octyl glucoside using a slow dilution-dialysis method [20,22]. The binding of Cθ, a modified θ-toxin, to these liposomes was analyzed at 10°C. Scatchard analysis of Cθ binding to the liposomes shows that the mode of toxin binding depends on the cholesterol content of the liposomes (Fig. 1). Scatchard plots of Cθ binding to liposomes containing 41.0 and 36.4 mol% cholesterol are curvilinear (Fig. 1A), suggesting that these liposomes contain at least two classes of toxin-binding sites. The dissociation constants for the high- and low-affinity toxin-binding sites are 2.7 and 170 nM for SOPC/POPG liposomes containing 41 mol% cholesterol and 1.6 and 130 nM for those containing 36.4 mol% cholesterol, respectively. The values are similar to those for intact cells [18,19] and for DSPC/DSPG/cholesterol (30–35 mol%) liposomes [20].

On the other hand, SOPC/POPG liposomes containing 34.9 mol% or less cholesterol give linear plots (Fig. 1A and B), showing that these liposomes contain only one class of toxin-binding sites. The dissociation constants for these liposomes are 130–270 nM, corresponding to the low-affinity binding sites. Under the same conditions, no detectable Cθ was bound to SOPC/POPG liposomes containing no cholesterol or to liposomes containing 42.6 mol% 4-cholesten-3-one, a cholesterol analogue with a 3-ketone instead of the 3β-OH. This indicates that the toxin binds specifically to cholesterol in liposomes, and not to phospholipids or to 4-cholesten-3-one. The results are consistent with the observation that θ-toxin and related thiol-activated cytolysins recognize only cholesterol or cholesterol analogues that have a 3β-OH group [6,10,20].

The number of high- and low-affinity toxin-binding sites was calculated from the Scatchard analyses and plotted against the mole percentage of cholesterol in the liposomes (Fig. 2A). High-affinity toxin-binding sites appear only in liposomes that contain 36 mol% or more cholesterol and their number rises steeply with increasing mole percentages of liposomal cholesterol (Fig. 2A, closed circles). In contrast, low-affinity toxin-binding sites are detected even in liposomes containing 10 mol% cholesterol, although the number of binding sites decreases to less than 1/100 as the liposomal

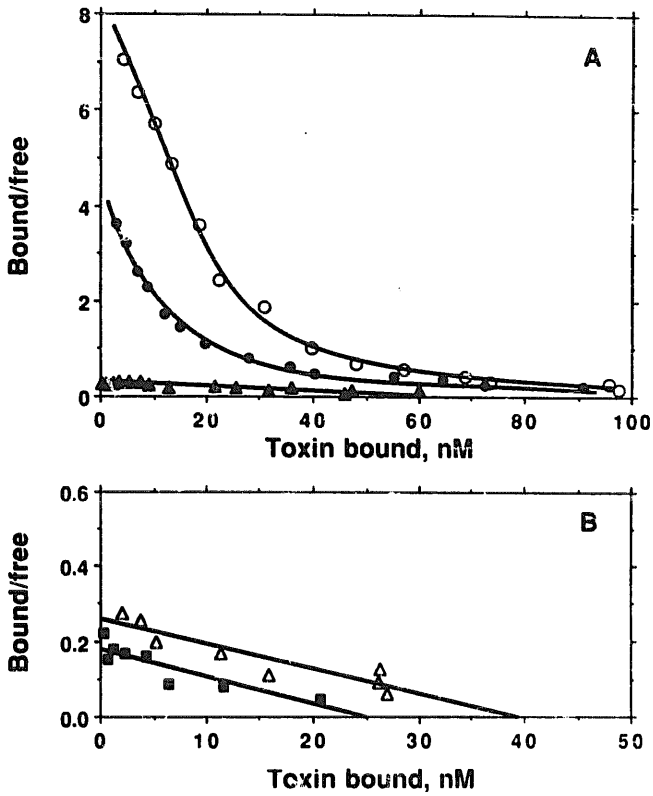

Fig. 1. Scatchard analysis of Cθ binding to various SOPC/POPG/cholesterol liposomes. An aliquot (1.1 ng) of ^{125}I-Cθ mixed with various amounts of unlabeled Cθ was incubated with SOPC/POPG liposomes containing 41.0 (○), 36.4 (●), 34.9 (▲), 24.8 (△) or 19.7 (■) mol% cholesterol in a 0.1 ml reaction mixture in the presence of phosphate-buffered saline and 0.5 mg/ml bovine serum albumin. The amounts of liposomes added to the reaction mixtures were varied so as that each reaction mixture contained 0.24 μg (A) or 12 μg (B) of liposomal cholesterol. After incubation for 3 h at 10°C, the liposomes were sedimented in a Beckman TLA 100 rotor at 80000 rpm for 10 min, and the radioactivities of both the supernatant and the pellet were measured in a gamma counter to determine the percentage of Cθ bound to the liposomes. The dissociation constants and the number of high- and low-affinity binding sites were estimated by computer analysis of the Scatchard plots as described in a previous paper [18]. The data are representative of three independent experiments.

cholesterol level decreases from 41 to 10 mol% (Fig. 2A, open circles).

It has been reported that phospholipid membranes containing 20 mol% or less cholesterol undergo phase transition between the gel and liquid-crystalline states at the phase transition temperatures of the individual phospholipids [1,2]. The phase transition temperature of SOPC is reported to be 6°C [23]. To exclude the possibility that phase transition might affect toxin binding to liposomes with low cholesterol contents, the relationship between toxin binding and liposomal cholesterol content was measured using MCθ at 25°C, a temperature well above the phase transition temperatures of the contained phospholipids. Essentially the same results were obtained at 25°C (Fig. 2A, triangles).

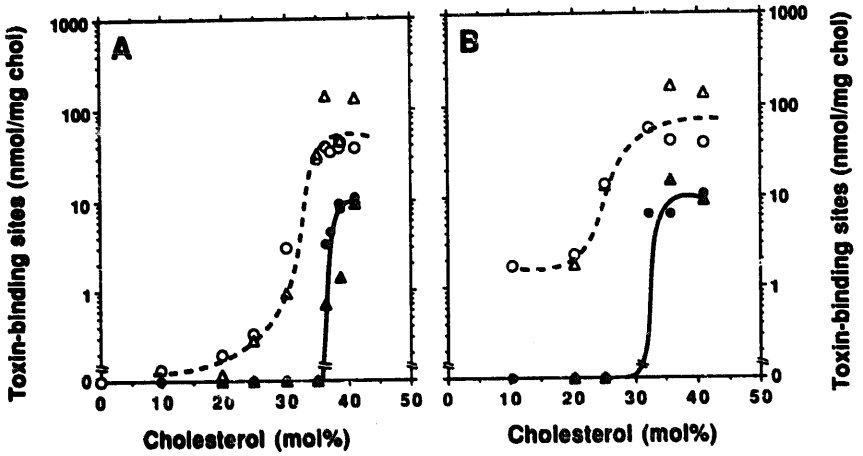

Fig. 2. Effect of mole percentage of liposomal cholesterol on toxin binding. Cθ (○,●) and MCθ (△,▲) were incubated with SOPC/POPG/cholesterol (A) and SOPC/POPG/cholesterol/4-cholesten-3-one (B) liposomes for 3 h at 10°C (○,●) or for 90 min at 25°C (△,▲) and the binding of the toxins to the liposomes was measured as described in the legend to Fig. 1. The number of high-(●,▲) and low-affinity (○,△) toxin-binding sites in each kind of liposome was obtained by Scatchard analysis and plotted against the mole percentage of cholesterol in the liposomes. Total sterols (cholesterol plus 4-cholesten-3-one) were 40–41 mol% in all SOPC/POPG/cholesterol/4-cholesten-3-one liposomes to give a constant sterol/phospholipid ratio.

as at 10°C, suggesting that the influence of phospholipid phase transition on toxin binding can be disregarded.

Next, we examined whether the coexistence of other sterols might affect the topology of cholesterol in liposomes. For this purpose, 4-cholesten-3-one was chosen since it does not itself bind to the toxin. A series of SOPC/POPG/cholesterol/4-cholesten-3-one liposomes (SOPC/POPG = 82:18, mol/mol, cholesterol + 4-cholesten-3-one = 40–41 mol%) was prepared and toxin binding was examined (Fig. 2B). Although the sterol (cholesterol plus 4-cholesten-3-one)/phospholipid ratio was kept constant in all the SOPC/POPG/cholesterol/4-cholesten-3-one liposomes, high-affinity toxin-binding sites were not detected in liposomes containing 25 mol% or less cholesterol (Fig. 2B, closed circles). If the state of cholesterol depends on the mole percentage of total sterols in the membrane, then liposomes containing 20 mol% cholesterol plus 20 mol% 4-cholesten-3-one should have half as many high-affinity sites as liposomes containing 40 mol% cholesterol. However, Fig. 2B shows that it is not the case. Similar results were obtained for low-affinity toxin-binding sites. In addition, the relationship between the number of toxin-binding sites and the cholesterol mole percentage is similar between liposomes with and without the cholesterol analogue (compare Fig. 2A and B), suggesting that the cholesterol analogue does not affect the topology of liposomal cholesterol.

Since Cθ and MCθ do not cause membrane lysis, the toxins are expected to bind only to cholesterol located in the outer leaflet of the lipid bilayer. Therefore, the distribution of cholesterol between the outer and inner leaflets of the liposomal bilayer might be one of the determining factors for the number of toxin-binding sites. This possibility was examined by measuring the susceptibility of liposomal cholesterol to cholesterol oxidase, an enzyme that attacks only cholesterol located in the outer leaflet of the bilayer [24]. However, the absolute amount of cholesterol oxidized by cholesterol oxidase is a linear function of cholesterol content; the percentage of oxidized cholesterol is independent of the cholesterol mole percentage in the

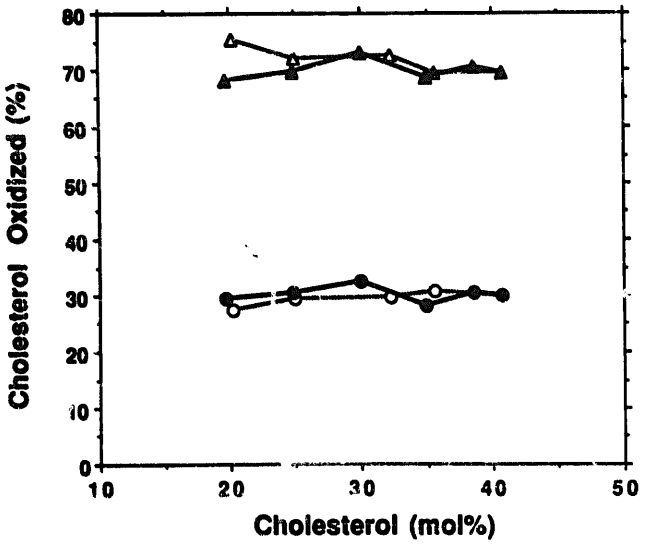

Fig. 3. Susceptibility of liposomal cholesterol to cholesterol oxidase. SOPC/POPG liposomes containing various mole percentages of cholesterol with (○,△) or without (●,▲) 4-cholesten-3-one were incubated with cholesterol oxidase for 3 h at 10°C (○,●) or for 90 min at 25°C (△,▲). Oxidation of liposomal cholesterol to 4-cholesten-3-one was measured as described in Materials and Methods. Total sterols (cholesterol plus 4-cholesten-3-one) were 40–41 mol% in all liposomes containing 4-cholesten-3-one (○,△).

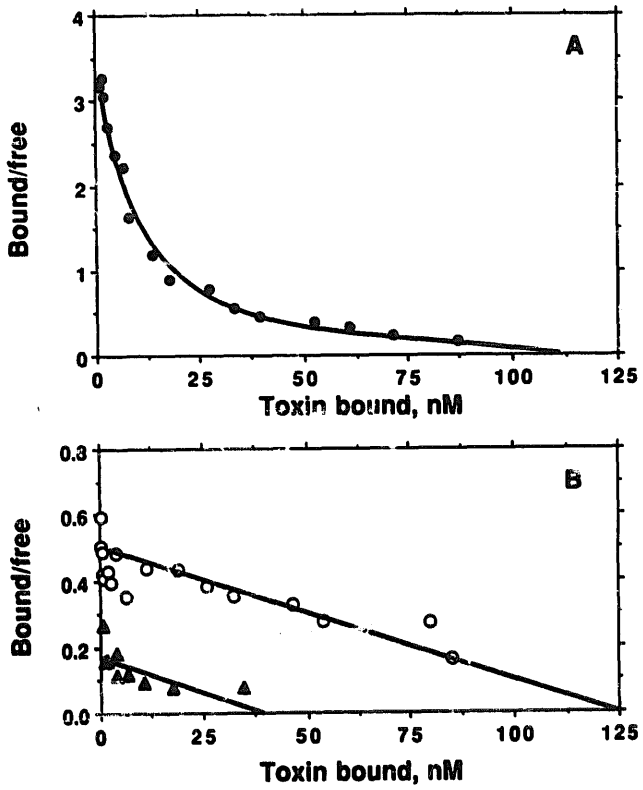

Fig. 4. Scatchard analysis of Cθ binding to EPC/EPG/cholesterol liposomes. Cθ was incubated with EPC/EPG (82.18, mol/mol) liposomes containing 40.3 (●), 36.1 (○), and 31.9 (▲) mol% cholesterol for 90 min at 10°C and the binding of Cθ was measured as described in the legend to Fig. 1. The amounts of liposomes added to the reaction mixtures were varied so as that each reaction mixture (0.1 ml) contained 0.24 μg (A) or 3.96 μg (B) of liposomal cholesterol. The data are representative of three independent experiments.

liposomes (Fig. 3). This suggests that the distribution of cholesterol between the outer and inner leaflets of the liposomal bilayer does not differ significantly among liposomes with different cholesterol contents. In contrast, the number of toxin-binding sites is not a linear function of cholesterol content; high-affinity toxin-binding sites appear only in liposomes containing 36 mol% or more cholesterol, and the number of low-affinity sites increases not linearly but exponentially in accordance with the increase in liposomal cholesterol (Fig. 2A). Therefore, the distribution of cholesterol in the outer and inner leaflets of the bilayer can not explain the mode of toxin binding.

Toxin binding to EPC / EPG / cholesterol liposomes

Next we examined whether the dependence of the number of high-affinity toxin-binding sites on the cholesterol mole percentage in SOPC/ POPG/ cholesterol liposomes applies to liposomes containing phospholipids with different fatty-acid compositions. Liposomes composed of egg PC (EPC), PG transesterified from egg PC (EPG), and various mole percentages of cholesterol were prepared. The binding of Cθ to the liposomes was measured at 10°C, a temperature higher than the phase transition temperatures of any of the phospholipids (−15 ~ −7°C) [25]. The Scatchard plot for Cθ binding to liposomes composed of EPC/EPG and 40.3 mol% cholesterol is curvilinear (Fig. 4A), indicating that the liposomes contain both high- and low-affinity toxin-binding sites. On the other hand, the plots for EPC/EPG liposomes containing 36.1 and 31.9 mol% cholesterol are linear (Fig. 4B), showing that these liposomes have only low-affinity toxin-binding sites. No detectable Cθ was bound to EPC/EPG liposomes containing no cholesterol, indicating that the binding is specific for cholesterol. The numbers of high- and low-affinity toxin-binding sites on the liposomes were calculated from the Scatchard plots (Fig.

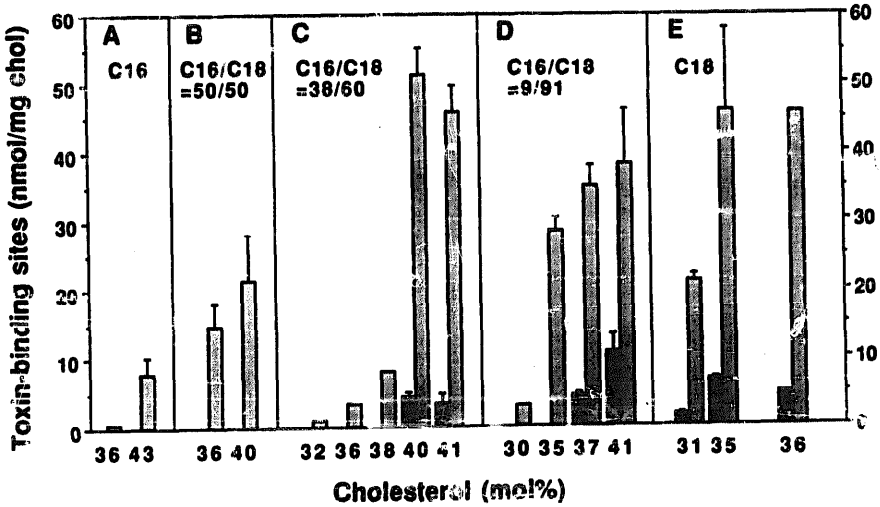

Fig. 5. Effect of the C-16/C-18 ratio of liposomal phospholipids on the number of toxin-binding sites. Cθ was incubated at 10°C with DPPC/DPPG (A), POPC/POPG (B), EPC/EPG (C), SOPC/POPG (D), DSPC/DSPG (E, the first two pairs of columns), and SOPC/dioleoyl-PG (E, the third pair of columns) liposomes containing the various cholesterol mol% shown on the horizontal axis, and the binding of Cθ was measured as described in the legend to Fig. 1. The numbers of high-affinity (solid bars) and low-affinity (stippled bars) toxin-binding sites were calculated from the Scatchard plots. The phosphatidylcholine/phosphatidylglycerol ratio of each type of liposome is 82:18 (mol/mol).

5C). High-affinity toxin-binding sites appear only in liposomes containing 40 mol% or more cholesterol. These results show that the number of high-affinity toxin-binding sites depends strictly on the mole percentage of cholesterol in both SOPC/POPG/cholesterol (Figs. 1 and 2) and EPC/EPG/cholesterol liposomes (Figs. 4 and 5C).

Chain length of liposomal phospholipids and toxin binding

We previously demonstrated that the number of toxin-binding sites depends on the chain lengths of the phospholipids when liposomes with the same cholesterol content are compared [20]. Cθ preferentially binds to cholesterol in liposomes containing 18-carbon hydrocarbon chains (C-18), rather than 16- or 14-carbon hydrocarbon chains (C-16 or C-14) [20]. On the other hand, the number of high-affinity toxin-binding sites depends strictly on the mole percentage of cholesterol as demonstrated in the previous sections (Figs. 1 and 2). This suggests that the mole fraction of liposomal phospholipids containing C-18 among the total phospholipids, especially the C-18/(C-16 + C-18) ratio in the case of naturally occurring phospholipids, might affect the dependence of high-affinity toxin-binding sites on cholesterol. To examine this possibility, toxin-binding sites in liposomes with various C-16/C-18 molar ratios were compared.

The C-16/C-18 molar ratio for EPC/EPG/cholesterol liposomes is estimated to be 38:60 based on fatty-acid composition [26], while that for SOPC/POPG/cholesterol liposomes is 9:91. These two types of liposomes differ in their threshold values for cholesterol mole percentage above which high-affinity toxin-binding sites appear (Fig. 5C and D). The threshold value for EPC/EPG/cholesterol liposomes is 40 mol% cholesterol (Fig. 5C), while that for SOPC/POPG/cholesterol liposomes is 36 mol% (Fig. 2A), suggesting that a decrease in the C-16/C-18 ratio causes a decrease in the threshold value. In fact, in distearoyl-PC (DSPC)/distearoyl-PG (DSPG)/cholesterol liposomes, which contain only C-18, high-affinity sites appear at 31 mol% cholesterol (Fig. 5E, and Ref. 20), much lower than the threshold values for the former two types of liposomes. In contrast, dipalmitoyl-PC (DPPC)/dipalmitoyl-PG (DPPG)/cholesterol liposomes, which contain only C-16, and 1-palmitoyl-2-oleoyl-PC (POPC)/POPG/cholesterol liposomes whose C-16/C-18 ratio is 50:50 have only low-affinity toxin-binding sites, at least up to 43 mol% and 40 mol% cholesterol, respectively (Fig. 5A and B, and Ref. 20). These results indicate that the C-16/C-18 ratio of liposomal phospholipids correlates with the existence of high-affinity toxin-binding sites and strongly suggest that cholesterol molecules interacting with C-18, but not with C-16, might form the high-affinity toxin-binding sites.

In addition to the threshold values, the C-16/C-18 ratio correlates with the number of high-affinity toxin-binding sites. For instance, POPC/POPG (82:18, mol/mol, C-16/C-18 = 50:50) liposomes containing 38 mol% cholesterol have no high-affinity toxin-binding sites, while SOPC/POPG liposomes (C-16/C-18 = 9.91) with the same cholesterol content have 6.7 nmol of high-affinity toxin-binding sites per mg of liposomal cholesterol. On the other hand, liposomes composed of POPC/SOPC/POPG (41:41:18, mol/mol, C-16/C-18 = 30:70) and 38 mol% cholesterol have 3.6 nmol of high-affinity sites, approximately half the number in SOPC/POPG/cholesterol liposomes (Fig. 6A). Thus, the greater the mole fraction of C-18, the more high-affinity sites appear when liposomes with the same cholesterol contents are compared (Figs. 5 and 6A).

Effects of phospholipid composition on toxin binding

The above results were obtained using liposomes composed of PC/PG (82:18) and cholesterol. The effects of phospholipid composition on toxin binding were next examined. First, the effect of PE was investigated by comparing Cθ binding to liposomes composed of EPC/EPE/EPG (72:10:18, mol/mol) and cholesterol with those composed of EPC/EPG (82:18) and cholesterol (Fig. 6D). EPE, PE transesterified from EPC, has the same fatty-acyl composition as EPC and was used as the source for PE to eliminate the effect of hydrocarbon chain length. Neither liposomes with nor without PE have high-affinity toxin-binding sites at 32 mol% cholesterol (Fig. 6D). At 36 mol% cholesterol, liposomes containing PE have high-affinity sites and a larger number of low-affinity sites than those without PE. At 40 mol% cholesterol the numbers of high- and low-affinity toxin-binding sites in the PE-containing liposomes are slightly larger than in those lacking PE. Thus, the existence of high-affinity toxin-binding sites depends on the cholesterol mole percentage in liposomes containing PE as well as in those without PE. PE decreases the threshold value of cholesterol above which high-affinity toxin-binding sites appear from 40 to 36 mol%, and increases the number of toxin-binding sites when liposomes with the same cholesterol content are compared.

Next, toxin binding to liposomes containing SM, rather than PC, was examined. To minimize the differences in the chain lengths of the fatty-acyl moieties of the liposomal phospholipids, liposomes containing *N*-oleoyl-SM were compared with those containing 1-myristoyl-2-oleoyl-PC (MOPC), POPC and SOPC. The numbers of high-and low-affinity toxin-binding sites in liposomes composed of *N*-oleoyl-SM/POPG (82:18, mol/mol) and 40 mol% cholesterol are closest to those in liposomes composed of POPC/POPG/40 mol%

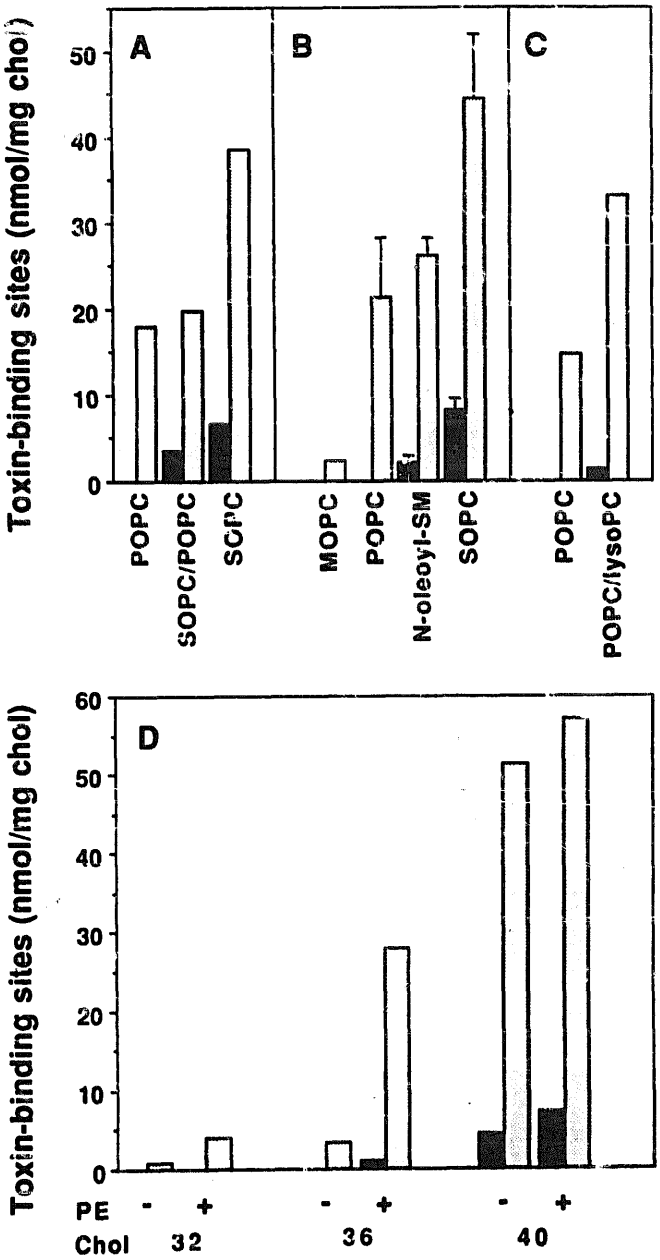

Fig. 6. Effects of the phospholipid composition of liposomes on toxin binding. The binding of Cθ to liposomes with various phospholipid compositions was measured as described in the legend to Fig. 1 and the numbers of high-affinity (solid bars) and low-affinity (stippled bars) toxin-binding sites were calculated from Scatchard plots. (A) Binding to liposomes composed of POPG/38 mol% cholesterol and either POPC, POPC/SOPC (1:1, mol/mol), or SOPC (PC/PG = 82:18, mol/mol). (B) Comparison of binding to *N*-oleoyl-SM/POPG/40 mol% cholesterol liposomes with that to MOPC/POPG/40 mol% cholesterol, POPC/POPG/40 mol% cholesterol, and SOPC/POPG/39 mol% cholesterol liposomes (PC/PG = 82:18, mol/mol). (C) Effect of lysoPC. Toxin binding to liposomes containing POPC/monopalmitoyl-PC/POPG (77:5:18, mol/mol) and 36 mol% cholesterol was compared with binding to POPC/POPG/36 mol% cholesterol liposomes (PC/PG = 82:18, mol/mol). (D) Effect of PE. The binding of Cθ to EPC/EPE/EPG (72:10:18, mol/mol) liposomes containing 32, 36 and 40 mol% cholesterol (Chol) was compared with Cθ binding to EPC/EPG (82:18, mol/mol) liposomes with the same cholesterol contents.

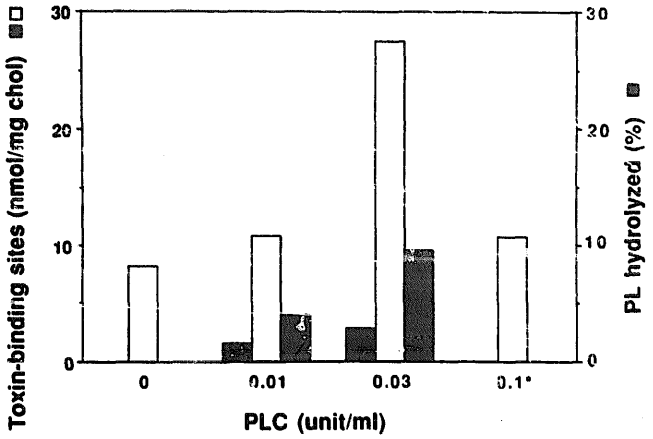

Fig. 7. Toxin binding to EPC/EPG/38 mol% cholesterol liposomes treated with phospholipase C (PLC). Liposomes containing 120 μg/ml cholesterol were incubated with either 0–0.03 unit/ml PLC or 0.1 unit/ml heat-inactivated PLC (shown as 0.1*) for 30 min at 25°C, and washed as described in Materials and Methods. The binding of Cθ to control and PLC-treated liposomes was measured as described in the legend to Fig. 1. The numbers of high-affinity (solid bars) and low-affinity (open bars) toxin-binding sites were calculated from Scatchard plots. Percentage of phospholipids hydrolyzed (stippled bars) was determined as described in Materials and Methods.

cholesterol among the three types of liposomes composed of PC/POPG and cholesterol, although the numbers of binding sites in SM-type liposomes are slightly larger than in POPC-type liposomes (Fig. 6B).

LysoPC in liposomes also affects toxin binding. POPC/monopalmitoyl-PC/POPG (77:5:18, mol/mol) liposomes containing 36 mol% cholesterol have high-affinity toxin-binding sites and a larger number of low-affinity sites than POPC/POPG (82:18) liposomes with the same cholesterol content (Fig. 6C). Removal of the phospholipid head groups from EPC/EPG/cholesterol liposomes (Fig. 7) or human red blood cells (data not shown) by treatment with phospholipase C increases both high- and low-affinity toxin-binding sites in parallel with the amount of phospholipid hydrolyzed.

Discussion

In this report we demonstrate that high-affinity toxin-binding sites appear only in liposomes with a high cholesterol content (Figs. 2, 5 and 6D). The threshold value for the cholesterol mole percentage above which high-affinity sites appear also depends on the chain length of the phospholipids (Fig. 5) and on phospholipid compositions (Fig. 6). The threshold values decrease as the mole fraction of C-18 increases among total phospholipids (Fig. 5). In addition, the higher the mole fraction of C-18, the more high-affinity sites appear when liposomes with the same cholesterol content are compared. These findings strongly suggest that

the mole percentage of cholesterol in combination with the chain length of phospholipids and other factors determines the topology of membrane cholesterol. In particular, the results suggest that cholesterol interacts with phospholipids with different chain lengths in different manners. It has been reported that for bilayers composed of saturated PCs with 12–16 carbons per chain, cholesterol increases membrane thickness by increasing the proportion of chains in the trans conformation [1,27]. On the other hand, cholesterol reduces the thickness of C-18 bilayers as the long phospholipid chains must deform or kink to accomodate the significantly shorter cholesterol molecule [1,27]. This observation also suggests that the interaction of cholesterol with C-18 may differ from that with C-16 or chains with fewer carbons.

Since the results above suggest that cholesterol molecules that interact with C-18 might form high-affinity toxin-binding sites, the relationship between the cholesterol/C-18 ratio and toxin binding was further analyzed. We postulated that cholesterol molecules that interact with C-18, but not those that interact with C-16, form high-affinity toxin-binding sites. In fact, DPPC/DPPG/cholesterol liposomes, which contain solely C-16, have no high-affinity toxin-binding sites, at least up to 43 mol% cholesterol (Fig. 5A). Since it has been reported that cholesterol can be incorporated into the structure of aqueous lamellar phospholipids up to 50 mol% [2], the cholesterol/phospholipid and cholesterol/phospholipid fatty-acyl chain ratios are maximally 1 : 1 and 1 : 2 (mol/mol), respectively. Cholesterol that interacts with C-18 is indicated as Chol* hereafter and in Fig. 8. If cholesterol interacts evenly with C-18 and C-16, the molar ratio of Chol* to C-18-phospholipids (Chol*/$(C\text{-}18)_2$) is equal to the molar ratio of cholesterol/phospholipid (Chol/PL). The relationship between the number of high-affinity toxin-binding sites and Chol/PL is obtained by combining the results in Figs. 2, 5 and 6A and is shown in the inset in Fig. 8. High-affinity toxin-binding sites appear only in liposomes with high Chol/PL, however, the plotted data are rather scattered depending on the liposomal phospholipids (Fig. 8, inset). However, if we make the assumptions that cholesterol in liposomes containing both C-16 and C-18 interacts with C-16 at a 1 to 2 molar ratio without producing high-affinity sites, and that the residual cholesterol interacts with C-18, a good correlation is observed between the number of high-affinity toxin-binding sites and Chol*/$(C\text{-}18)_2$ (Fig. 8). High-affinity sites appear only in liposomes whose Chol*/$(C\text{-}18)_2$ is 0.4 or more and their number correlates well with Chol*/$(C\text{-}18)_2$ regardless of phospholipid chain length. These results strongly suggest that high-affinity sites are formed by cholesterol in regions consisting of cholesterol–C-18 phospholipids where the cholesterol to C-18 phospholipid ratio is above 0.5.

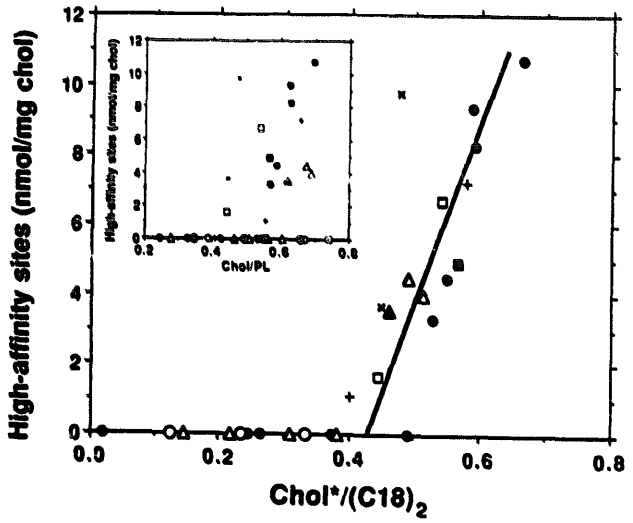

Fig. 8. Relationships between high-affinity toxin-binding sites and the cholesterol and phospholipid compositions of liposomes. The number of high-affinity toxin-binding sites in various liposomes are plotted against the cholesterol/phospholipid (Chol/PL, mol/mol) ratio (inset) or against the ratio of cholesterol interacting with C-18 (Chol*) to 2×C-18 (shown as Chol*/$(C\text{-}18)_2$ on the horizontal axis, mol/mol). Details are described in the text. x, dioleoyl-PC/dioleoyl-PG/cholesterol; □, DSPC/DSPG/cholesterol; ■, SOPC/dioleoyl-PG/cholesterol; ●, SOPC/POPG/cholesterol; △, EPC/EPG/cholesterol; ▲, SOPC/POPC/POPG/cholesterol; ○, POPC/POPG/cholesterol and POPC/dioleoyl-PG/cholesterol; ⊙, DPPC/DPPG/cholesterol; +, EPC/EPE/EPG/cholesterol liposomes.

How does the cholesterol–C-18 phospholipid interaction produce the high- and low-affinity toxin-binding sites? It might be speculated that cholesterol in the region consisted of 1 to 1 molar ratio of cholesterol to C-18 phospholipid is more flexible than that in the region consisted of 1 to 2 molar ratio or less of cholesterol to C-18 phospholipid. This means that the toxin can access easier to the former region than the latter region, resulting in the formation of high- and low-affinity sites.

Since cholesterol is reported to have the weakest affinity for PE among phospholipids [28], one might assume that cholesterol interacts with liposomal phospholipids other than PE. Accepting this assumption, the data for high-affinity sites in EPC/EPE/EPG/cholesterol liposomes (shown as + in Fig. 8) closely fit the regression line in Fig. 8. Thus, the increase in high-affinity sites in PE-containing liposomes might be explained by the exclusion of cholesterol from PE domains with a consequent increase in the Chol*/$(C\text{-}18)_2$ ratio. Previous reports suggest that hydrogen bonding between the amino group of PE and the phosphate of a neighboring phospholipid [29] and poor hydration of the surface of PE membranes [30] might cause the exclusion of cholesterol from PE domain.

In addition to PE, SM and lysoPC in liposomes also affect toxin binding. However, it is primarily the cholesterol mole percentage in liposomes that determines the number of high-affinity toxin-binding sites since SM- and lysoPC-containing liposomes with low cholesterol contents have no high-affinity sites (data not shown). θ-Toxin binds neither to phospholipids developed on TLC plates [18,19] nor to liposomes containing phospholipids but no cholesterol. Therefore, these phospholipids must affect the binding affinity of θ-toxin to cholesterol by modifying the topology of cholesterol in membranes. There are minor phospholipids containing fatty-acyl chains longer than 18 carbon atoms in cell membranes. The effect of such very long fatty-acyl chains on toxin binding remains to be elucidated. Preliminary experiments show that replacement of 1/3 of the POPC in POPC/POPG/cholesterol liposomes with 1-palmitoyl-2-arachidonoyl-PC has no effect on toxin binding.

θ-Toxin and related thiol-activated cytolysins share common properties with respect to their protein structures and modes of action. The cytolysins recognize only cholesterol or analogues with a 3β-OH group [6,10,20]. The presence of an α-OH group on C-3, esterification of the hydroxyl group, or its substitution with keto group renders the sterol inactive for the toxin binding [6]. In contrast to cholesterol the thiol analogue of cholesterol (thiocholesterol) did not interact with streptolysin O, one of the thiol-activated cytolysins, indicating a strict specificity of the oxygen atom of the 3β-OH group for toxin interaction [31]. Our data showing that liposomes containing 4-cholesten-3-one instead of cholesterol have no toxin-binding sites indicate that the 3β-OH group of cholesterol is essential to constitute both high- and low-affinity toxin-binding sites. In addition to the 3β-OH group, since sterols with no aliphatic side chain, such as dehydroepiandrosterone and estradiol-17β, show no inhibitory effects on the lytic activity of θ-toxin and related cytolysins [10,32], the aliphatic side chain of cholesterol has been suggested to constitute toxin-binding sites [6,10].

Some cholesterol antibodies were reported which react with liposomes containing 71 mol% cholesterol, where cholesterol presumably exists in aggregates, but not with those containing 43 mol% cholesterol [33]. This suggests the possibility that cholesterol in aggregates or in dispersion might differ in orientation from cholesterol interacting with phospholipids. Cholesterol inhibits lytic activity of θ-toxin when given as cholesterol dispersion [6,32] as well as given as liposomes composed of cholesterol and phospholipids. In addition, θ-toxin binds to cholesterol in dispersion and forms polymers, being observed as ring- and arc-shaped structure by electron microscopy [13,34]. These observations suggest that cholesterol in aggregates have toxin-binding sites as well as cholesterol interacting with phospholipids, although no quantitative data are available for binding affinity of the toxin to cholesterol in aggregates.

θ-Toxin and related cytolysins appear in polymeric form, on cell membranes [13,15,34]. We have previously shown that such polymerization of θ-toxin on cell membranes is an essential step in the lytic process [15,18,19], because θ-toxin remains in the monomer form on membranes when hemolysis is inhibited by the addition of θ-toxin fragment [15]. On the other hand, membrane-bound MCθ, a derivative of θ-toxin with the same binding activity as θ-toxin but no hemolytic activity [18], remains in the monomer form as judged by electron microscopy [18] and by sucrose density gradient analysis [15]. In addition, Cθ, another θ-toxin derivative that causes no hemolysis below 20°C [17,19], remains in the monomer form below 20°C. Thus, Cθ and MCθ appear to be good probes for membrane cholesterol since they do not cause the redistribution of membrane cholesterol upon adsorption onto the cell membrane. θ-Toxin and related cytolysins, however, cause membrane disorder as filipin and saponins do [7,8].

We have previously reported that intact cells, such as human and sheep erythrocytes and lymphoma BALL-1 cells, have both high- and low-affinity toxin-binding sites on their plasma membranes [18,19]. Preliminary experiments with human erythrocytes from different subjects show that the number of high-affinity toxin-binding sites increases in accordance with the increase in cholesterol mole percentage (Ohno-Iwashita, Y., Iwamoto, M. and Ideguchi, Y., unpublished data), suggesting a correlation between cholesterol content and the number of high-affinity toxin-binding sites in intact cells. In general, the ratio of cholesterol to other plasma membrane lipids is about 0.6–0.8 (mol/mol) [35–37]. Taking the phospholipid fatty-acid composition and PE content of plasma membranes into account, plasma membranes in a wide variety of cells might have both high- and low-affinity toxin-binding sites. On the other hand, the ratio of cholesterol to phospholipid in intracellular membranes is reported to be less than that in plasma membranes: 0–0.2 in both mitochondrial membranes and endoplasmic reticulum, and 0.3–0.5 in both Golgi apparatus and lysosomal membranes [35,37]. Liposomes whose cholesterol/phospholipid ratio is 0.4 or less have no high-affinity toxin-binding sites and very few low-affinity sites (Figs. 2 and 8). Thus, judging by toxin binding, the topology of membrane cholesterol in intracellular membranes is expected to be different from that in plasma membranes. It would be also interesting to know the difference in the topology of membrane cholesterol between apical and basolateral plasma membranes, since their ratio of cholesterol to phospho-

lipid has been reported to be quite different [38]. It is noteworthy that θ-toxin distinguishes some specific populations of membrane cholesterol as high- and low-affinity binding sites that are indistinguishable based on cholesterol oxidase susceptibility (Fig. 3 and Ref. 20) or on desorption rates from membranes [20]. From this viewpoint, the use of modified θ-toxin could provide a unique tool for the study of cholesterol organization in biological membranes, such as for the detection of cholesterol distribution in subcellular organelles and plasma membranes. Fluorescent staining of cholesterol in cell membranes and intracellular organelles using Cθ and MCθ coupled with fluorescent dyes is now under investigation.

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