Evidence That Clustered Phosphocholine Head Groups Serve as Sites for Binding and Assembly of an Oligomeric Protein Pore*

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High susceptibility of rabbit erythrocytes toward the pore-forming action of staphylococcal α-toxin correlates with the presence of saturable, high affinity binding sites. All efforts to identify a protein or glycolipid receptor have failed, and the fact that liposomes composed solely of phosphatidylcholine are efficiently permeabilized adds to the enigma. A novel concept is advanced here to explain the puzzle. We propose that low affinity binding moieties can assume the role of high affinity binding sites due to their spatial arrangement in the membrane. Evidence is presented that phosphocholine head groups of sphingomyelin, clustered in sphingomyelin-cholesterol microdomains, serve this function for α-toxin. Clustering is required so that oligomerization, which is requisite for stable attachment of the toxin to the membrane, can efficiently occur. Outside these clusters, binding to phosphocholine is too transient for toxin monomers to find each other. The principle of membrane targeting in the absence of any genuine, high affinity receptor may also underlie the assembly of other lipid-inserted oligomers including cytotoxic peptides, protein toxins, and immune effector molecules.

Staphylococcal α-toxin, archetype of a pore-forming cytolytin, is secreted as a water-soluble, 34-kDa monomer that binds to target membranes and then oligomerizes to form membrane-inserted heptameric channels (1, 2). The three-dimensional structure of the monomer can be deduced from the recently solved structure of the related staphylococcal LukF leukocidin (3), and the structure of the heptamer is also known (4). Data obtained by Bayley and co-workers (1, 6, 7) and our-selves (2, 5) have led to a consensus model of pore assembly. Membrane-bound monomers interact with each other to form homotypic oligomeric prepores that initially lack permeabilizing activity. Early prepores are stable in non-denaturing detergents but unstable in SDS. Cooperative conformational changes transform the early to the late prepores, which resists dissociation in SDS at 25 °C. The transmembrane channel is formed when the pore-forming amino acid sequence finally inserts into the bilayer to create an amphipathic membrane-spanning β-barrel (8–11).

One major unresolved question relates to the molecular nature of the physiologically relevant binding site on target cells. This issue is surrounded by paradoxes. Mammalian cells differ widely in their susceptibility to permeabilization by α-toxin, classic extremes being represented by rabbit and human erythrocytes. The former are lysed by nanomolar concentrations of the toxin, whereas lysis of human erythrocytes first occurs at 200-fold higher concentrations. Binding studies have revealed the existence of high affinity, saturable interaction sites on rabbit cells that are absent on human erythrocytes. However, both cell types additionally have low affinity binding sites, which are responsible for lysis of the “resistant” human cells at high toxin concentrations (12). All efforts to identify a classic receptor on susceptible target cells have failed. The issue is further confounded by the fact that liposomes and lipid bilayers composed solely of phosphatidylcholine are highly susceptible to permeabilization by α-toxin (13). Hence, a protein or glycolipid receptor is not required for the toxin to bind to membranes and form channels in the first place.

Watanabe et al. (14) have considered that phosphatidylcholine might be the ligand for α-toxin. This suggestion derived from the observation that phosphocholine could inhibit toxin-dependent permeabilization of liposomes, albeit only at very high concentrations of ≥20 mM. According to the crystal structure of leukocidin, members of this toxin family indeed possess a putative binding site for phosphocholine (3). Thus, we are faced with another paradox that a molecule destined to interact with α-toxin apparently does so with an affinity that is so low as to render a biological relevance improbable.

Here, a concept is advanced that provides an explanation for the past findings. It is proposed that, by clustering, low affinity binding moieties can assume the role of high affinity “receptors” because they render rapid toxin oligomerization possible. In the absence of clustering, binding of toxin monomers will be too transient to support oligomerization at low concentrations of toxin. Phosphocholine head groups fulfill this function in the case of α-toxin, and their clustering on susceptible cells occurs through the association of sphingomyelin with cholesterol. Sphingomyelin-cholesterol-rich microdomains represent operational units in which clusters of...
proteins and lipids coordinately exert their functions (15–17). Interaction with clustered low affinity binding sites may underlie the physiological action of other oligomerizing toxins and effector molecules of the immune system.

**EXPERIMENTAL PROCEDURES**

**Materials**—Egg yolk phosphatidylcholine (PC),

2 egg yolk phosphatidylethanolamine (PE), brain sphingomyelin (SM), ceramide, and cholesterol were obtained from Sigma (Deisenhofen, Germany). Production of wild-type α-toxin and the active mutant G130C and labeling of the latter with fluorescein-5-maleimide or with biotin-maleimide was as described (18). Carrier-Free Na\(^{22}\)I was from Amersham Biosciences (Freiburg, Germany), and wild type α-toxin was radioiodinated as described (12). \(^{3}H\) Choline chloride was from PerkinElmer Life Sciences (Cologne, Germany). Kits for determining ATP and cholesterol were from Roche Diagnostics (Mannheim, Germany).

**Cell Culture**—THP-1 cells were grown in RPMI 1640 medium (Invitrogen), supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, streptomycin (100 μg/ml), and penicillin (100 μg/ml). Jurkat cells were grown in Iscove’s modified Dulbecco’s medium (Invitrogen), supplemented with 10% FCS, 20 mM Hepes, streptomycin (100 μg/ml), and penicillin (100 μg/ml). Human skin fibroblast cultures were established from surgical foreskin specimens from patients. Pieces of skin were minced and cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% FCS, 2 mM glutamine, and streptomycin–penicillin–streptomycin for 2–3 weeks. Cells were passaged several times in 250-ml tissue culture flasks (Greiner). Cultures between the fourth and eighth passages were used in the experiments.

CHO cells (LY-B and LY-B/cLCB1) were cultivated as described (19, 20). The mutant cells LY-B completely lacked *de novo* synthesis of sphingolipids. Transfection of these cells with a recombinant plasmid expressing cLCB1 (LY-B/cLCB1) complemented the defect (19–21). The sphingomyelin content is reduced to less than 30% following culture of either cell type in sphingomyelin-free medium (19–21). Ham’s F-12 medium supplemented with 10% fetal calf serum, penicillin (100 μg/ml), and streptomycin (100 μg/ml) was used as the normal culture medium. Nutridoma medium (F-12 medium containing 1% Nutridoma SP medium (Roche Diagnostics) and gentamycin (10 μg/ml)) and Nutridoma BO-medium (Nutridoma medium supplemented with 0.1% FCS and 10 μM sodium oleate/albumin (Sigma)) were used as sphingomyelin-deficient medium. The revertant cells (LY-B/cLCB1) can replenish their sphingomyelin content in Nutridoma BO medium, whereas the mutant cells cannot. There are no appreciable differences in the contents of other major phospholipids or cholesterol in these cells (19–21). CHO cells were routinely maintained in the normal culture medium in a 5% CO\(_2\) atmosphere at 37 °C. For culture in different media, the mutant and revertant cells were seeded into a 100-mm Petri dish containing 5 ml of normal culture medium and incubated at 37 °C for 2 h and washed twice with phosphate-buffered saline. Then cell monolayers were cultured in Nutridoma medium with 10% FCS to normalize their sphingomyelin content or in Nutridoma BO-medium or in Nutridoma-medium for 3 days at 37 °C, 5% CO\(_2\).

**Lysis Inhibition by Phosphocholine**—α-Toxin was preincubated in a microtiter plate with increasing concentrations of phosphocholine in a total volume of 100 μl of HBSS for 30 min, 22 °C. One-hundred microliters of a rabbit erythrocyte suspension (5 × 10\(^8\)/ml) were then added to each well. After 1 h, 22 °C, the plates were centrifuged, and hemolysis was quantified by measurement of hemoglobin absorption at 412 nm.

**Cholesterol Depletion**—Cholesterol extraction from rabbit erythrocytes was performed using phosphatidylcholine liposomes (22, 23). Rabbit erythrocytes (4 × 10\(^8\) cells/ml) were incubated with 1 mM phosphatidylcholine liposomes at 37 °C. After 3 h, the cells were sedimented and washed five times with HBSS.

Cholesterol extraction from nucleated cells was achieved using methyl-β-cyclodextrin (Sigma). THP-1 cells (4 × 10\(^6\) cells/ml) were incubated with 10 mM methyl-β-cyclodextrin in DMEM for 30 min, 37 °C, and washed three times with DMEM. Half of the cells were used for binding or spectrofluorometric studies; the other half were washed in HBSS, and a cholesterol determination (Roche Diagnostics) was performed.

**Labeling of Phosphocholine**—Fibroblasts were cultured in the presence of \(^{3}H\) choline chloride (1 μCi per culture dish) in DMEM with 1% FCS at 37 °C, 5% CO\(_2\) for 3 h. Cells were washed three times with DMEM and treated with 0.1 unit/ml *Bacillus cereus*-phospholipase C (Calbiochem, Bad Soden, Germany) or with 10 mM methyl-β-cyclodextrin in DMEM. Cell-associated radioactivity and radioactivity in cell supernatants were determined after 30 min, 37 °C.

**Determination of Sphingomyelin and Phosphatidylcholine**—Cells were seeded, incubated in the normal culture medium for 1 day, and after washing with serum-free medium were cultured in Nutridoma medium with 10% FCS or in Nutridoma BO-medium or sphingomyelin-free Nutridoma medium for 3 days at 37 °C. The cells were prelabeled with \(^{3}H\) choline (1 μCi/ml, overnight; PerkinElmer Life Sciences). At the end of the incubation, cells were washed and harvested by scraping, lipids were extracted with chloroform/methanol (ratio 2:1) (24), and unlabeled sphingomyelin and phosphatidylcholine were added as internal standards. Phospholipids were separated by thin-layer chromatography on silica 60 gel plates (Merck, Darmstadt, Germany) using chloroform/methanol/acetic acid (65/25/10 v/v/v) as solvent and stained by iodine vapor, and the spots corresponding to sphingomyelin and phosphatidylcholine were scraped off and counted for radioactivity in a liquid scintillation counter.

**Liposome Preparation**—Lipid films containing 1 mg of total lipid in different molar ratios were dried down from chloroform/methanol (2:1 v/v) under nitrogen and then rehydrated in 1 ml of HBSS, pH 7.5. Liposomes containing sphingomyelin were rehydrated at 45 °C, while the others were rehydrated at room temperature. Liposomes were rapidly frozen (−70°C acetone bath) and thawed (45°C water bath) six times.
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freeze-thawing, liposomes were passed through a 0.1-μm polycarbonate filter (Nuclepore, Clifton NJ) 10 times, using a Mini-extruder (Avanit Polar Lipids, Alabaster, AL). The sphingomyelin liposomes were extruded at 45 °C, and the others were extruded at room temperature.

Quantitation of α-Toxin Binding—Binding studies using erythrocytes were performed with the radioiodinated α-toxin as described (12). Fluorescein-labeled G130C α-toxin was used in binding studies with nucleated cells and liposomes.

THP-1 cells or CHO cells were incubated with 30 nM fluorescein-labeled G130C α-toxin for 1 h at 37 °C. After washing, cells were resuspended in 1 ml of HBSS, and bound toxin was quantified by spectrofluorometry. Fluorescence emission spectra were recorded in a Spex Fluoromax fluorometer (wavelength, excitation: 488 nm, emission: 500–550 nm, scanning interval: 1 nm). The spectra of appropriate blanks (buffer or cell suspension) were recorded and subtracted from the sample spectra.

In the liposome experiments, membrane-bound and free toxin were separated by flotation. Fluorescein-labeled α-toxin was incubated with the liposomes for 2 h, 25 °C, and sucrose was added to 25% (w/v). 3-ml samples were applied to the bottom of centrifuge tubes and were overlaid with 1 ml of 20% sucrose, 1 ml of 15% sucrose, and 0.2 ml of HBSS. The liposomes were floated to the top of the gradient by centrifugation overnight in an SW50.1 swing-out rotor (150,000 × g, 4 °C), and the lipid-associated fluorescence was analyzed by spectrofluorometry and SDS-PAGE.

In the kinetic experiments, liposomes with 40% cholesterol, either 10% PC or 10% SM, and supplemented with 25% of PE and phosphatidylserine (PS) each were mixed with pyrene-labeled toxin (mutant S3C) in a stopped-flow apparatus (SF-61-DX2, HiTech, Salisbury, UK). The excitation wavelength was set to 335 nm. Emitted light was collected employing a filter DX2, HiTech, Salisbury, UK). The excitation wavelength was set to 488 nm, emission: 500–550 nm, scanning interval: 1 nm. The spectra of appropriate blanks (buffer or cell suspension) were recorded and subtracted from the sample spectra.

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Non-denaturing PAGE—The native gel electrophoresis system described previously was used (26), with the exception that electrophoresis buffer contained 25 mM rather than 2.5 mM potassium phosphate buffer. In these experiments, a 1% suspension of rabbit erythrocyte ghosts was incubated with 30 nM of biotinylated G130C α-toxin in the presence or absence of unlabeled α-toxin. This revealed that the saturable, high affinity binding sites evidenced by the abrupt increase in binding of the radiotracer at high concentrations of non-labeled toxin.

RESULTS

Cholesterol Depletion Selectively Destroys the High Affinity Binding Site for α-Toxin on Rabbit Erythrocytes—Rabbit erythrocytes were suspended in buffer containing 15 mM dextran 4 to prevent hemolysis and depleted of cholesterol by incubation with liposomes (22, 23). This method was employed because rapid depletion with methyl-β-cyclodextrin led to high cellular fragility and spontaneous hemolysis. In contrast, use of liposomes led to slow depletion that could be terminated before the cells became too fragile. Cells containing 18 ± 5% of their original cholesterol content were used in binding studies. 50 μl (4 × 10⁵ cells/ml) of erythrocyte suspension were incubated with 70 pM ¹²⁵I-α-toxin in a total volume of 200 μl of HBSS, 15 mM dextran 4000, in the presence or absence of unlabeled α-toxin at the depicted concentrations. After 2 h, 22 °C, the amount of bound radiotracer was determined. Note the absence of the saturable, high affinity binding sites for α-toxin in the cholesterol-depleted cells and persistence of the low affinity binding sites evidenced by the abrupt increase in binding of the radiotracer at high concentrations of non-labeled toxin.

When cholesterol-depleted rabbit erythrocytes were employed, a completely different mode of toxin binding was observed that duplicated the results previously obtained with human erythrocytes. Then, no binding of the toxin tracer was observed in the absence or presence of low concentrations of unlabeled toxin. This revealed that the saturable, high affinity binding sites had been lost. When the concentration of unlabeled toxin was increased above 10⁻⁷ M, binding of the radioactive tracer recurred. This again was as previously described (12) and demonstrated the presence of a second population of non-saturable binding sites.

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FIGURE 1. Cholesterol depletion leads to selective loss of high affinity binding sites on rabbit erythrocytes. Rabbit erythrocytes were depleted of cholesterol by incubation with phosphatidylcholine liposomes for 3 h, and cells containing 18 ± 5% of their original cholesterol content were used in binding studies. 50 μl (4 × 10⁵ cells/ml) of erythrocyte suspension were incubated with 70 pM ¹²⁵I-α-toxin in a total volume of 200 μl of HBSS, 15 mM dextran 4000, in the presence or absence of unlabeled α-toxin at the depicted concentrations. After 2 h, 22 °C, the amount of bound radiotracer was determined. Note the absence of the saturable, high affinity binding sites for α-toxin in the cholesterol-depleted cells and persistence of the low affinity binding sites evidenced by the abrupt increase in binding of the radiotracer at high concentrations of non-labeled toxin.
High Affinity Interaction of α-Toxin with THP-1 Cells Is Abrogated Both by Cholesterol Extraction and by Sphingomyelinase Treatment—In the experiment of Fig. 2, THP-1 cells were either depleted of cholesterol by incubation with methyl-β-cyclodextrin (27, 28), or they were treated with sphingomyelinase or with phospholipase C from B. cereus (4). Sphingomyelinase, but not B. cereus phospholipase C, cleaves sphingomyelin in biological membranes (29). α-Toxin was applied at 30 nM so that binding would occur only via high affinity interaction. As shown in Fig. 2, treatment with methyl-β-cyclodextrin or with sphingomyelinase led to marked reduction in α-toxin binding, whereas binding was not affected by treatment with phospholipase C.

To ascertain that sphingomyelinase and methyl-β-cyclodextrin exerted distinct effects, fibroblasts were pulsed with [3H]choline to label the phosphocholine head groups. As shown in Table 1, sphingomyelinase, but not methyl-β-cyclodextrin, provoked loss of the marker to the cell supernatants. Cholesterol determinations confirmed that sphingomyelinase treatment did not lead to reduction of cellular cholesterol. When phospholipase C was applied, it was found that this enzyme, which cleaves phosphatidylcholine but not sphingomyelin (29), did not remove appreciable amounts of phosphocholine from the intact cells (Table 1). This concurred with early work showing that phosphatidylcholine headgroups are not readily accessible to B. cereus phospholipase C in biological membranes (29).

Reduction of toxin binding was mirrored by abrogation of cytotoxicity, both cholesterol-depleted and sphingomyelinase-treated cells withstood incubation with 30 nM α-toxin without loss of ATP for 2 h. However, when toxin doses were raised to >200 nM, membrane permeabilization occurred in all cells (data not shown).

Millimolar Concentrations of Phosphocholine Compete for Binding of α-Toxin to Rabbit Erythrocytes—The lysis inhibition experiments reported by Watanabe et al. (14) employed lecithin liposomes as targets, but analogous experiments using susceptible cells have not been performed. In the experiment of Fig. 3, hemolytic titration of α-toxin was performed in the presence of increasing concentrations of phosphocholine. It was found that phosphocholine inhibited binding of α-toxin to the high affinity binding sites, with 50% lysis inhibition being observed at ~0.6 mM of the ligand.

Reduced Binding of α-Toxin to Sphingomyelin-deficient Cells—To directly test whether phosphocholine head groups of sphingomyelin might indeed participate in binding α-toxin, experiments were conducted in CHO mutant cells (LY-B) and revertant cells (LY-B/cLCB1). The results of sphingomyelin determinations in these cells are shown in Table 2. The sphingomyelin content was normal in both cells following incubation in FCS. Sphingomyelin content was reduced by ~70% when either cell line was cultured in sphingomyelin-free medium. In contrast, culture in Nutridoma BO medium led to normalization of sphingomyelin content in the revertant cells, whereas the content in the mutant LY-B cells remained low.

Fig. 4 shows the respective toxin binding data. Equivalent amounts of toxin became bound to revertant (R) or mutant (M) cells following culture in FCS (A). After selective replenishment of sphingomyelin in the revertant cells (B), these bound normal amounts of α-toxin, whereas binding to the mutant cells was approximately halved. In the absence of sphingomyelin replen-
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Sphingomyelin and Cholesterol Together Generate the Binding Site for α-Toxin in Liposomes—Fluorescein-labeled α-toxin was applied at 30 nM to liposomes containing an equimolar mix of PE and PS with varying supplements. As shown in Fig. 5A, addition of either 40 mol % cholesterol or 20 mol % sphingomyelin led to no appreciable toxin binding. However, binding occurred when both cholesterol and sphingomyelin were present. When the latter liposomes were treated with sphingomyelinase, the binding site disappeared. The essential role of phosphocholine became further apparent from the lack of toxin binding to liposomes supplemented with cholesterol and 20% ceramide. These findings indicated that the phosphocholine head groups of sphingomyelin most likely represented the toxin binding sites but that they needed to be clustered through aggregation with cholesterol to fulfill their function.

To corroborate this contention, PS/PE liposomes containing 40 mol % cholesterol were supplemented with 5 mol % phosphatidylcholine or 5 mol % sphingomyelin. As shown in Fig. 5B, only liposomes containing cholesterol + sphingomyelin bound α-toxin; phosphatidylcholine was not effective. However, when the phosphatidylcholine concentration was raised to 40 mol %, binding activity appeared (Fig. 5B). This binding was found to no longer depend on the presence of cholesterol. We concluded that at the latter concentration, the phosphocholine head groups are densely enough distributed on the membrane to support toxin oligomerization independent of cholesterol.

Evidence That α-Toxin Molecules Bind in Clusters on the Membrane—The collective results pointed to the possibility that α-toxin might be binding primarily to clustered phosphocholine head groups, and this might allow the toxin monomers to immediately oligomerize at the binding sites. To test this, rabbit erythrocyte membranes were exposed to 30 nM α-toxin on ice for 1, 5, or 10 min, washed, and solubilized in Triton X-100. Native PAGE was performed in deoxycholate to differentiate monomers from the detergent-stable precipitates and pores. As shown in Fig. 6 (left panel), this electrophoresis system allowed distinction between toxin monomers and oligomers (lanes 1 and 3). When native α-toxin was added to Triton solubilisates, no spontaneous formation of oligomers occurred (lane 2). When α-toxin was allowed to bind for just 1–10 min on ice, however, all detectable membrane-bound toxin was recovered in oligomeric form (lanes 4 – 6). Lateral diffusion of molecules in erythrocyte membranes is known to be markedly retarded at 0°C (30), and these results therefore were interpreted to indicate that the α-toxin monomers did primarily reside in immediate proximity with each other on the membranes and could thus rapidly form oligomers. The right panel shows an SDS gel of the first three samples and sample 6 of the native gel. As expected, the monomeric samples (lanes 1 and 2) and the control oligomer sample (lane 3) ran true in the SDS gel. In contrast, the sample containing the deoxycholate-stable oligomer (lane 6, left panel) generated the monomeric toxin band in the SDS gel. This confirmed that the deoxycholate-stable oligomer forming at 0°C represented a prepore.

To monitor the kinetics of oligomer formation, we performed stopped-flow experiments using pyrene-labeled S3C (25) toxin. Pyrene can exhibit fluorescence around 475 nm if the molecules come close together and form excimers. This occurs upon oligomerization of α-toxin (25). The results of an experiment performed with liposomes containing 10% PC or 10% SM are shown in Fig. 7. When PC-containing liposomes were employed, essentially no formation of excimers was observed over a time period of 10 min. In contrast, excimer formation was readily observed with SM liposomes and could already be detected within 1 s of incubation and essentially without a lag phase. This experiment unequivocally showed that, when binding of α-toxin occurs, the oligomers form virtually instantaneously.
DISCUSSION

A simple concept can now be formulated to explain how a protein can efficiently be targeted to cell membranes in the absence of a conventional, high affinity receptor. We propose that, in line with the crystal structure of both the monomer of LukF (3) and the oligomer of \( \alpha \)-toxin (4), \( \alpha \)-toxin has a low affinity binding site. Liposomes (PE,PS) were complemented with 40 mol % cholesterol and 5% sphingomyelin (column 1); 5 mol % phosphatidylcholine (column 2); 40 mol % sphingomyelin (column 3); and 40 mol % phosphatidylcholine (column 4). Binding of \( \alpha \)-toxin was determined as described for A. When present at 5 mol %, only sphingomyelin supported toxin binding to the cholesterol-containing liposomes.
affinity binding site for phosphocholine. This assumption received experimental support from the finding that millimolar concentrations of phosphocholine successfully competed for binding of the toxin to rabbit erythrocytes. Oligomerization is required for stable membrane binding and pore formation to occur. This takes a long time if the low affinity binding sites are dispersed on the membrane unless high toxin doses are applied. The situation changes when the binding molecules are clustered. Then, toxin monomers have a much higher probability to be close to each other, and oligomerization can occur almost instantaneously (Fig. 8). Two key findings supported this contention. First, α-toxin was recovered in oligomerized form in cell membranes following just very brief binding periods of 1–10 min on ice. Second, stopped-flow experiments revealed virtually instantaneous oligomerization of the toxin upon incubation with 10% SM/cholesterol liposomes, as detected by excimer formation of pyrene-labeled molecules. This did not occur when 10% PC/cholesterol liposomes were employed. In these experiments, dipalmitoylphosphatidylcholine or distearoylphosphatidylcholine could also not replace SM, and no excimer formation was observed (data not shown). In fact, fluorescence resonance energy transfer experiments indicate that in contrast to SM, dipalmitoylphosphatidylcholine forms no clusters in the presence of 40% cholesterol (31, 32).

Binding experiments complemented these findings by showing that little binding of the toxin occurred when liposomes with low PC content were employed. When the PC concentration was raised, binding could take place, in accord with the idea that phosphocholine head groups needed to be clustered so that toxin oligomers could instantaneously form.

It is notable that, although phosphatidylcholine is the major phospholipid in the outer membrane leaflet of mammalian plasma membranes, the head groups are not uniformly available for binding by extracellular proteins. This is reflected by the fact that the mammalian phosphocholine-binding, C-reactive protein also cannot extensively bind to intact cells (33). Furthermore, many phospholipases C are unable to penetrate into the plasma membrane bilayer because the lateral pressure is too high (29, 34, 35). These facts have long been known but the implications are not widely appreciated. We confirmed that phospholipase C from B. cereus, which specifically cleaves phosphatidylcholine (29), was unable to remove appreciable amounts of phosphocholine from fibroblasts. Possibly, phosphocholine groups are available for saturation with membrane-inserting proteins in predominantly sphingomyelin-cholesterol-microdomains at the surface of nucleated cells. That sphingomyelin-cholesterol microdomains indeed represented the “specific” binding sites for α-toxin received support from two independent cell experiments. First, both cholesterol extraction and sphingomyelinase treatment destroyed the high affinity binding sites on cells. Of note, sphingomyelinase-treated cells do not lose their membrane cholesterol to intracellular pools (36). Second, a mutant cell line with reduced sphingomyelin content (~30% of normal cells) bound ~50% less α-toxin. This experiment was particularly informative because revertant cells were available that could be cultured under two different conditions, one leading to normal and the other leading to reduced sphingomyelin content. Satisfactorily, correlation with α-toxin binding was observed. These experiments were complemented by studies with liposomes, which continued to corroborate the concept. Thus, binding and heptamerization occurred when α-toxin was applied to liposomes containing cholesterol and sphingomyelin but not when either cholesterol or sphingomyelin was absent. Sphingomyelinase also destroyed the binding site on liposomes. Moreover, ceramide, which lacks the phosphocholine head group, could not substitute for sphingomyelin. Finally, phosphatidylcholine also could not functionally replace sphingomyelin in this liposomal system if present in low concentrations.

All our findings would thus be most consistent with the notion that the phosphocholine head group represents the binding moiety for α-toxin within membrane microdomains. Very satisfactorily, the single substitution toxin mutant R200C was previously noted to have lost binding capacity (37). Arg^{200} is one residue that lines the putative phosphocholine binding pocket (3, 4).

The current concept would account for all the puzzling findings in the literature regarding the elusive nature of the receptor for α-toxin. It accounts for the discrepancy between susceptibility of lecithin liposomes to toxin action with the widely varying susceptibility of mammalian cells and explains why a protein or glycolipid receptor has never been found. The observation that liposomes with a high content of phosphatidylcholine are susceptible to low doses of α-toxin has always been an enigma. Now, the finding would be explained by the need of obtaining sufficiently dense clusters of phosphocholine head groups at the membrane surface for high affinity binding to take place. Cholesterol-sphingomyelin domains or clustered phosphocholine head groups do not exist in prokaryotes, and this would explain why these organisms are not vulnerable to attack by physiological concentrations of α-toxin. Conversely, varying susceptibilities of different cell species toward α-toxin is likely governed by the number of clustered phosphocholine head groups that are exposed to the extracellular environment. Finally, irreversibility of α-toxin binding to cells as observed in the previous study (12), which was not consistent with the classical receptor concept, is now also explained.
The proposed model may apply to many situations in which water-soluble proteins need to oligomerize to insert into membranes. Lyssin, the pore-forming toxin from the coelomic fluid of an earthworm, has been reported to preferentially bind to SM/cholesterol liposomes (38). Aerolysin, another oligomerizing pore-forming toxin, reportedly binds to glycosylphosphatidylinositol-anchored proteins (39, 40). Perfringolysin, prototype of a cholesterol-binding pore former, associates with raft lipids (41). In this case, cholesterol might analogously function to provide the location for clustering and oligomerization of the toxin. Perforin, the pore-forming molecule of cytotoxic lymphocytes, has also been reported to bind to phosphocholine with low affinity (42). It will be of interest to discern in the future whether the concept emerging from the present study is indeed extendable to these and other agents that form oligomeric pores.

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