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Staphylococcus aureus alpha toxin can bind to cholesterol-sensitive phosphatidyl choline head group arrangements

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Abstract
Bacterial pore-forming toxins form the largest class of bacterial toxins and constitute an important family of virulence factors. *Staphylococcus aureus* produces several pore-forming toxins with different target cell specificity, one of which is alpha-toxin (α-toxin or alpha hemolysin). While pore-forming toxins are generally thought to bind to unique specific receptors at the surface of target cells, α-toxin may have different modes of binding. We here provide evidence that α-toxin may also bind directly to cholesterol-sensitive phosphatidylcholine head group assemblies. Specifically, we show that polarized cells are sensitive to α-toxin only when exposed on the basolateral side and that channel formation by α-toxin is inhibited upon cholesterol removal and enhanced by the absence of glycosphingolipids. Our data is consistent with the binding to cholesterol-sensitive assemblies of phosphatidylcholine.

Introduction
Bacterial pore-forming toxins are major virulence factors produced by a great variety of pathogenic bacteria [1]. They are produced as soluble monomeric proteins that, upon binding to the target cell membrane, oligomerize into ring-like macromolecular structures that subsequently insert into the plasma membrane and alter membrane permeability. Binding to the target cells is generally mediated by specific receptors that can be proteins, lipids, or sugars. The lipid bilayer, its composition, and lipid distribution also play important roles [2]. Specific arrangement of lipids such as sphingomyelin-cholesterol clusters may actually constitute the receptors of certain pore-forming toxins [3].

Objective
Staphylococcus aureus is a major human pathogen that leads to thousands if not millions of infections every year around the world, with sometimes fatal outcomes. The bacterium secretes a variety of pore-forming toxins that target different cell types largely due to their ability to bind to differentially expressed receptors [4]. The exact nature of the receptor of *S. aureus* α-toxin (α-toxin or alpha hemolysin) has remained some what a debate. Studies in the 1980s pointed towards a requirement of phosphocholine-containing lipid head groups for binding and activity of α-toxin using artificial liposomes [5]. In 2011, α-toxin was shown to bind the transmembrane metalloprotease ADAM10 [6]. Here we investigated the effect of α-toxin on polarized epithelial cells and found that binding, and thereby activity, is restricted to the basolateral surface. We further present evidence that is consistent with the ability of α-toxin to bind to cholesterol-dependent phosphocholine head group assemblies.

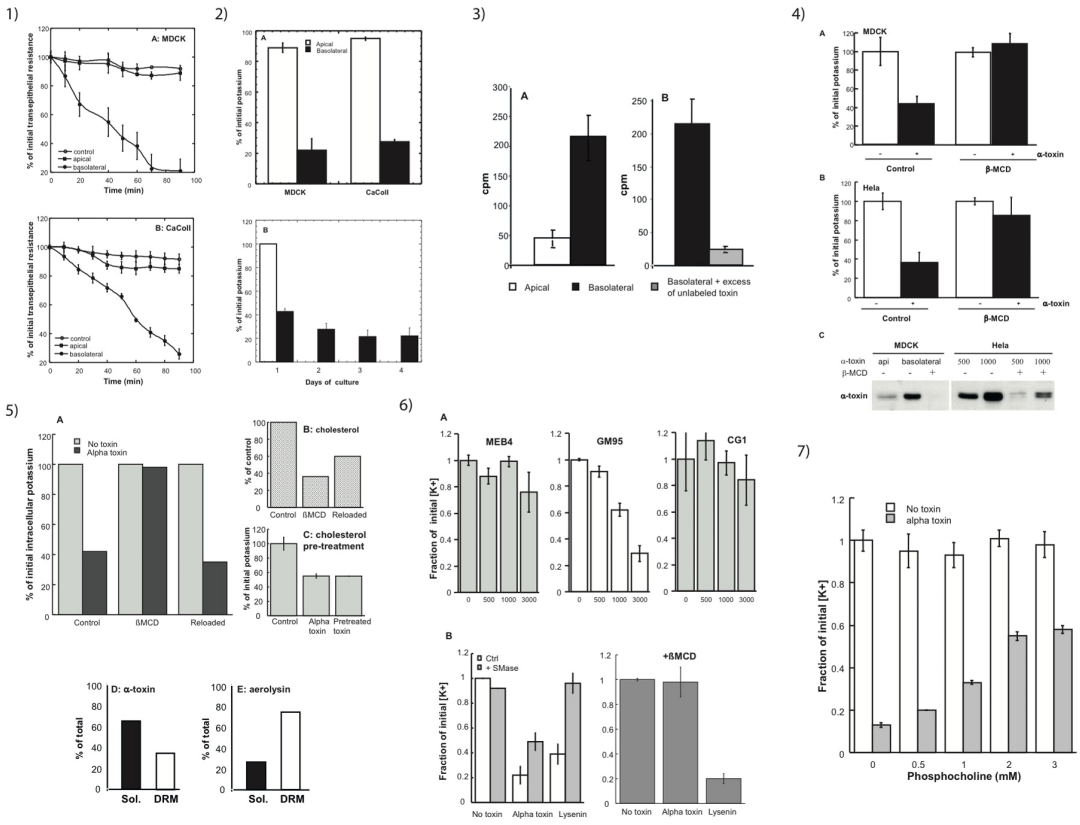

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Figure Legend

Figure 1.

Polarized MDCK or CaCoII cells were incubated with 500 ng/ml α-toxin in complete medium at 37°C either on the apical side or on the basolateral side. At the indicated time, the transepithelial resistance was measured with a Millipore Millicell resistance meter and expressed as a percentage from the starting measure at T=0. The error bars indicate standard deviations (n>3). 500 ng/ml α-toxin leads to a drop in TER when the toxin was added from the basolateral side.

Figure 2.

Upper panel: Polarized MDCK or CaCoII cells were incubated with 500 ng/ml α-toxin for 1 h at 37°C in complete medium either on the apical side or on the basolateral side of the monolayer. The intracellular content of potassium was measured and expressed as a fraction of the initial potassium content. Potassium content was released when the toxin was added from the basolateral side. The error bars indicate standard deviations (n>3). Lower panel: Non-polarized MDCK cells were platted at day 0 at high dilution. At different days following plating, cells were incubated or not with 500 ng/ml α-toxin for 1 h at 37°C in complete medium. The intracellular content of potassium was measured and expressed as a fraction of the initial potassium content. Potassium content was released already after 1 day of platting. Error bars indicate standard deviations (n=3).

Figure 3.

Polarized MDCK cells were incubated with 500 ng/ml iodinated α-toxin for 4 h at 37°C in complete medium either on the apical side or on the basolateral side of the monolayer, with and without 2 ug/ml excess unlabeled α-toxin. The cells were lysed and the radioactivity was counted. Error bars indicate standard deviations. (N=3).

Figure 4.

(A and B) Non-polarized MDCK or HeLa cells were incubated with 500 ng/ml α-toxin for 1 h at 37°C in serum-free medium with or without a pretreatment of 30 min of 10 mM β-methyl-cyclodextrin. The intracellular content of potassium was measured and

expressed as a fraction of the initial potassium content. The error bars indicate standard deviations (n=3). **(C)** The binding of 1 h at 37°C of 500 ng/ml α-toxin was observed by western blotting on polarized MDCK cells and on HeLa cells with or without a pretreatment of 30 min of 10 mM β-methyl-cyclodextrin in serum-free medium.

Figure 5.

(A) Non-polarized MDCK were incubated or not with 500 ng/ml α-toxin for 1 h at 37°C in serum-free medium with or without a pretreatment for 30 min 10 mM β-methyl-cyclodextrin and reloaded or not with 0.3 mM cholesterol. The intracellular content of potassium was measured and was expressed as a fraction of the initial potassium content. Data represents the mean of two highly reproducible independent experiments. **(B)** The amount of extracted and reloaded cholesterol on MDCK was estimated by TLC. Cholesterol could be reloaded to 67±6% of control cells (*n*=3). **(C)** Non-polarized MDCK were incubated or not with 500 ng/ml α-toxin for 1 h at 37°C in serum-free medium with or without a pretreatment of the toxin with 0.3 mM cholesterol. The intracellular content of potassium was measured and was expressed as a fraction of the initial potassium content. The error bars indicate standard deviations (*n*=3).

Figure 6.

(A and B) HeLa cells were incubated with 500 ng/ml iodinated α-toxin or 100 ng/ml iodinated aerolysin for 4 h at 37°C in complete medium. Cells were solubilized at 4°C in 1% Triton X-100 and soluble fractions were separated from DRMs after centrifugation. (n=1).

Figure 7.

(A) Glycoshingolipid-deficient mouse melanoma cell line GM95, the parental cell line MEB4, and cell line transfected with ceramide glucosyltransferase to restore glycolipid synthesis of CG1 were incubated with different concentrations of α-toxin (in ng/ml) for 1 h at 37°C in complete medium. The intracellular content of potassium was measured and expressed as a fraction of the initial potassium content. The error bars indicate standard deviations (*n*=3). **(B)** HeLa cells were incubated or not with 500 ng/ml α-toxin or 500 ng/ml Lysenin for 1 h at 37°C in serum-free medium with or without a pretreatment for 24 h with sphingomyelinase 1 U/ml or for 30 min with 10 mM β-methyl-cyclodextrin. The intracellular content of potassium was measured and expressed as a fraction of the initial potassium content. The error bars indicate standard deviations (*n*=3).

Figure 8.

HeLa cells were incubated or not with 500 ng/ml α-toxin for 1 h at 37°C in serum free medium with or without a pretreatment of 1 h of different concentration of phosphocholine. The intracellular content of potassium was measured and was expressed as a fraction of the initial potassium content. The error bars indicate standard deviations (n=3).

Cell Culture and toxin purification

MDCK and CaCoII cells were grown and maintained in Dulbecco's Modified Eagle's medium (1:1) (DMEM; Sigma Chemical Co., St. Louis) complemented with 10 % fetal calf serum, 2 mM L-glutamine, 4.5 g/l glucose under standard tissue culture conditions. All experiments on polarized cells were performed on COSTAR filter-grown cells. Unless specified, MDCK and CacoII cells were grown on filters for 4 and 12 days, respectively. Staphylococcal α-toxin was produced and purified as previously described and labeled ^{125}I using Iodogen reagent (Pierce) according to the manufacturer's recommendations. The radiolabeled toxin was separated from free iodine by gel filtration on a PD10-G25 column (Pharmacia, Sweden) equilibrated with phosphate buffered saline pH 7.4. Radiolabeled proaerolysin ran as a single band on a SDS-gel.

Transepithelial electrical resistance

Transepithelial electrical resistance (TER) was determined by applying an AC square wave current of ±20 μA at 12.5 Hz with a silver electrode and measuring the voltage deflection elicited with a silver/silver chloride electrode using an EVOM (World Precision Instruments). TER values were obtained by subtracting the contribution of the filter and bath in solution. TER changes upon toxin treatment were expressed as a percentage of TER at time 0. All experiments were performed at 37°C. For these experiments, cells were grown on filters with a 24 mm diameter. During the experiments, 1.5 ml IM was added to the apical compartment and 3 ml to the basolateral compartment. Cells were

incubated for 30 min in IM at 37℃ prior to the first TER measurement.

Potassium efflux measurements

Confluent monolayers grown on 24 mm filters were washed once and incubated in a medium (IM) containing Glasgow minimal essential medium (GMEM) buffered with 10 mM HEPES, pH 7.4, for 30 min at 37℃, in the absence of toxin. Monolayers were further incubated at 37℃ with α-toxin in IM for various times. Cells were subsequently washed with ice cold (pH 7.4) potassium-free choline medium containing 129 mM choline-Cl, 0.8 mM $MgCl_2$, 1.5 mM $CaCl_2$, 5 mM citric acid, 5.6 mM glucose, 10 mM NH_4Cl, 5 mM H_3PO_4 and solubilized with 0.5% Triton-X100 in the same buffer for 20 min at 4℃. The potassium content of the cell lysates was determined by flame emission photometry using a Philips PYE UNICAM SP9 atomic adsorption spectrophotometer.

Triton-X100 insolubility

Cells were treated with ^{125}I-α-toxin, scrapped from the filter, harvested by centrifugation and resuspended in 0.5 ml of cold buffer containing 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100 as well as cmplete, a cocktail of protease inhibitors (Roche). Membranes were solubilized by rotary shaking at 4℃ for 30 min. The detergent-insoluble fractions were obtained by high-speed centrifugation (30 min at 4℃ at 55, 000 rpm in a TLS55 Beckman rotor) as described previously [8].

Western blots

MDCK and HeLa cells were harvested, washed with PBS, and homogenized by passing through a 22G injection needle in HB (2.9 mM imidazole and 250 mM sucrose, pH 7.4) containing the Roche mini tablet protease inhibitor cocktail following manufacturer's instructions. Protein quantification was done with Pierce BCA kit. Proteins were loaded at 40 mg prot/lane and separated on a 4–20% acrylamide Novex gel under reducing conditions and transferred to nitrocellulose membranes (Schleicher and Schuell). Membranes were incubated with a polyclonal antibody against α-toxin (sigma, S7531).

Reagents

Sphingomyelinase from *Bacillus cereus* (Sigma, S9396) was used at 1 U/ml for 24 h prior to experiments (100 ul in a well from 12 wells plate). Methyl-beta-cyclodextrin (Sigma, M7439) was used at 10 mM 30 min at 37℃ in serum-free medium. Phosphocholine was purchased from Sigma (P0378) and used at the described concentration. **Results &**

Discussion

Polar effect of staphylococcal α-toxin on epithelial cells

To test whether MDCK and CaCoII cells were sensitive to α-toxin, we measured the effect of the toxin on the transepithelial resistance (TER) of cell monolayers. The toxin was added to filter grown cells either only to the apical or only to the basolateral compartment. To our surprise, both polarized cell types were sensitive to the toxin only when exposed on the basolateral side (Fig. 1).

To get a more direct estimate of the pore-forming activity of α-toxin on these polarized cells, we measured the change in intracellular potassium. If α-toxin forms a channel in the apical or basolateral plasma membrane, this should lead to potassium efflux across this membrane. Once more, α-toxin was added either apically or basolaterally to filter grown cells. As can be seen in figure 2 (upper panel), potassium efflux was significant only when α-toxin was added to the basolateral membrane of either MDCK or CacoII cells. The kinetics of channel formation were fast, since cells were essentially depleted of potassium within 1 h of exposure to a 500 ng/ml α-toxin concentration as shown for MDCK cells.

The basolateral membrane of a polarized epithelial cell is often considered as equivalent to the plasma membrane of a non-polarized cell, the apical membrane being considered more specialized. We therefore expected non-polarized MDCK cells to be sensitive to α-toxin. Cells were platted at high dilution (1/10) in order to prevent cell contacts, and potassium efflux measurements were performed. To our surprise, non-polar MDCK cells were insensitive to α-toxin (not shown because equal to controls). To test whether basolateral sensitivity required the full establishment of polarity, cells were platted on filters at a 1:1 dilution and the sensitivity to basolateral α-toxin was measured every day for 4 days. As shown in figure 2 (lower panel), 1 day after platting, MDCK cells were

already sensitive to basolateral α-toxin. These observations suggest that cell contact and formation of a tight monolayer, but not full polarization which requires approx. 4 days for MDCK cells, is required for MDCK cells to exhibit basolateral sensitivity to α-toxin.

Differential binding of α-toxin to the apical and basolateral membranes of MDCK cells

We next monitored whether binding occurred preferentially on the basolateral side. As shown in figure 3, binding to the apical side is far lower than binding to the basolateral side. Binding to the basolateral side was specific since it could be competed with a 50-fold excess of unlabeled toxin (Fig. 3B). The fact that a 50-fold excess was sufficient to inhibit binding by more than 80% also indicates that there were a limited number of binding sites.

Channel formation by α-toxin is inhibited upon cholesterol removal

It has previously been observed by us and by others that certain bacterial pore-forming toxin such as aerolysin and Clostridum α-toxin as well as related toxins such as *Helicobacter pylori* VacA or anthrax toxin protective antigen, preferentially associate with raft-like domains. To investigate whether the mode of action of Staphylococcal α-toxin also involves cholesterol-rich domains, we analyzed the effect of cholesterol depletion using β-methyl-cyclodextrin (β-MCD). Removal of cholesterol from the basolateral side of the MDCK monolayer led to a drastic inhibition of the α-toxin-induced potassium efflux (Fig. 4A; since β-MCD itself led to a decrease in intracellular potassium, results were expressed as a percentage of potassium at time 0). We then investigated what step in the mode of action was affected and found that binding of α-toxin to β-MCD-treated cells was essentially abolished (Fig. 4C). Since β-MCD could also affect cell polarity and thereby explain the loss of sensitivity, we tested the effect of cholesterol depletion by β-MCD on a non-polarized α-toxin-sensitive cell line, namely HeLa. As for MDCK cells, we found that β-MCD inhibited the toxin-induced potassium efflux (Fig. 4B) and toxin binding (Fig.4C).

The effect observed upon β-MCD treatment was due to the removal of cholesterol and not to the concomitant release of another important factor since reloading of cholesterol restored α-toxin sensitivity (Fig. 5A, B). These observations raise the possibility that as for certain cholesterol-dependent toxins (CDTs), cholesterol could be the receptors. This seems unlikely since preincubation of α-toxin with cholesterol did not affect the activity in agreement with previous observations on liposomes [5]. Pretreatment with cholesterol has been shown to inhibit pore formation by certain cholesterol-binding CDTs [7].

The cholesterol dependence of α-toxin binding raises the possibility that it binds to raft-like domains. As a rough estimate of this, we probed for the association of α-toxin with detergent-resistant membranes. Polarized MDCK cells were treated with α-toxin from the basolateral side, solubilized in Triton X-100 at 4°C and detergent-soluble material was separated by detergent-insoluble material by high-speed centrifugation [8]. As shown in figure 6, α-toxin was mainly associated with the detergent-soluble fraction in contrast to aerolysin, which was here used as a control [9], suggesting that unlike aerolysin, alpha toxin preferentially binds to detergent-soluble membranes.

Inhibition of glycosphingolipid synthesis leads to an increase in α- toxin sensitivity

To further explore the possibility that α-toxin binds to lipid rafts, we analyzed the effect of depleting cells of another major component, namely glycosphingolipids. For this we made use of a mouse melanoma cell line MEB4 and its glycolipid-deficient mutant GM95. GM95 has a defect in ceramide glucosyltransferase I (CerGlcTI) that catalyzes the first step of glycosphingolipid synthesis [10]. As shown in figure 7A, MEB4 cells were insensitive to α-toxins, but surprisingly GM95 cells showed sensitivity, which was lost upon recomplementation of the cells with the CerGlcTI gene (CG1 cells). Differences between these different cell lines could be partly due to charges in lipid composition, in addition to the lack of glycolipids. This behavior is similar to that observed for the earthworm pore-forming toxin lysenin [3]. Interestingly lysenin also shows a polarized effect on epithelial cells, with only the basolateral membrane being sensitive. α-toxin and lysenin, however, do not bind to the same sites at the target cell surface since we found no effect of cholesterol depletion on pore formation by lysenin (Fig. 7B, right

panel).
Lysenin is well known to bind to sphingomyelin [3], but to only special arrangements of sphingomyelin that are perturbed by the presence of glycosphingolipids (which would explain why these sphingomyelin arrangements are preferentially found on the basolateral membrane of polarized cells) [3]. It could be that α-toxin also binds to arrangements of sphingomyelin, yet different from those recognized by lysenin. α-toxin was indeed found not to bind to sphingomyelin vesicles unless cholesterol was present [5]. We therefore tested the effect of sphingomyelinase treatment on the α-toxin-induced potassium efflux. While sphingomyelinase treatment abolished the sensitivity of cells to lysenin, it only had a mild effect on the sensitivity to α-toxin (Fig. 7B, left panel), indicating that α-toxin does not bind to sphingomyelin, or to a pool of sphingomyelin that is not removed by the treatment.
Studies in the late 1980's pointed towards a requirement of phosphocholine-containing lipid head groups for α-toxin binding and activity using artificial liposomes [5]. The crystal structure of the α-toxin heptamer revealed the presence of a phosphocholine binding pocket between the rim and stem domains of the pore-forming complex [11]. To test the importance of phosphocholine-binding in pore-forming activity of target cells, we analyzed the effects of phosphocholine chloride on the toxin-induced efflux from HeLa cells, using choline chloride as a control. We found that increasing concentrations of phosphocholine led to a gradual inhibition of toxin activity (Fig. 8).

Conclusions

The present experiments show that polarized epithelial cells in culture such as MDCK or CacoII show a clearly polarized sensitivity to *S. aureus* α-toxin, only the basolateral membrane showing sensitivity. This sensitivity correlates to the ability of the toxin to bind. Binding was found to be specific, as indicated by a competition experiment, and highly sensitive to the presence of cholesterol, but occurred in non-raft-like membranes, i.e., fluid membranes. Consistent with this localization, binding was not mediated by sphingomyelin and even enhanced by the absence of glycolipids, which indeed preferentially localize to the apical plasma membrane of polarized cells. Combined with the presence of phosphocholine-binding sites in the α-toxin pore complex and with the inhibitory effect of soluble phosphocholine, the present experiments are consistent with the ability of α-toxin to bind to cholesterol-dependent assemblies of phosphatidylcholine. Thus depending of the body site, α-toxin may bind to ADAM10 or to phosphocholine head groups. We cannot exclude that binding of α-toxin to ADAM10 is cholesterol-dependent.

Limitations

Potassium efflux was used as a readout for pore formation. The known structure of the α-toxin pore indicates that pores formed by this toxin, lead to the flux of potassium. Pore formation may also trigger the opening of potassium channel, thus enhancing the apparent efflux.
The effect of β-MCD is extremely pleiotropic. The fact that sensitivity to α-toxin was restored upon cholesterol reloading suggests that the observed effects were due to the removal of cholesterol.
The sensitivity to α-toxin varies enormously between cells. This could be due to the relative presence of phosphatidyl choline arrangements or expression of other receptors such as ADAM10. Moreover, we found that the sensitivity to α-toxin also varied greatly by the passage number of tissue culture cells. At passage number above 6–7, HeLa cells became gradually insensitive to α-toxin. This could explain variability observed between different laboratories. At present, it is unclear how phosphocholine clusters could be specifically manipulated.

Additional Information

Methods

Cell Culture and toxin purification

MDCK and CaCoII cells were grown and maintained in Dulbecco's Modified Eagle's medium (1:1) (DMEM; Sigma Chemical Co., St. Louis) complemented with 10 % fetal calf serum, 2 mM L-glutamine, 4.5 g/l glucose under standard tissue culture conditions. All experiments on polarized cells were performed on COSTAR filter-grown cells. Unless specified, MDCK and CacoII cells were grown on filters for 4 and 12 days, respectively. Staphylococcal α-toxin was produced and purified as previously described and labeled ^{125}I using Iodogen reagent (Pierce) according to the manufacturer's recommendations. The radiolabeled toxin was separated from free iodine by gel filtration on a PD10-G25 column (Pharmacia, Sweden) equilibrated with phosphate buffered saline pH 7.4. Radiolabeled proaerolysin ran as a single band on a SDS-gel.

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Supplementary Material

Please see https://sciencematters.io/articles/201602000024.

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Ethics Statement

Not applicable.

Citations

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