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Different structural requirements for adenylate cyclase toxin interactions with erythrocyte and liposome membranes

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Abstract

The bifunctional *Bordetella* adenylate cyclase toxin-hemolysin (ACT) penetrates target cell membranes, forms cation-selective channels and subverts cellular signaling by catalyzing uncontrolled conversion of ATP to cAMP. While primarily targeting phagocytes expressing the $\alpha_M\beta_2$ integrin (CD11b/CD18), the toxin can also penetrate mammalian erythrocytes lacking the receptor and membrane endocytosis. We sought here to analyze the membrane interactions of ACT in a liposome model. Insertion of ACT into liposome membranes required calcium and caused leakage of entrapped fluorescent probes due to liposome disruption, as indicated by similar release kinetics for the ~398 Da FITC probe and its ~4400 Da dextran conjugate. However, the non-acylated proACT, which does not penetrate cellular membranes, exhibited higher capacity to bind and lyze liposomes than the mature toxin, showing that the fatty-acyl modification was not required for penetration of ACT into the lipid bilayer. Individual deletions within the channel-forming, acylation and repeat domains of ACT abolished its capacity to disrupt both liposomes and erythrocytes. In contrast to erythrocyte binding, however, the liposome binding was only lost upon a simultaneous deletion of both the channel-forming and acylation domains, suggesting that the acylation domain was also involved in liposome penetration of ACT. Moreover, substitutions of glutamates 509 and 516 by lysines, which strongly enhanced the channel-forming and hemolytic activity of ACT, did not affect its capacity to disrupt liposomes. This shows that the mechanism of ACT action in cellular membranes is not fully reproduced in liposome membranes.

Keywords: *Bordetella pertussis*; Adenylate cyclase toxin; Membrane interaction; Liposome; Mutant

1. Introduction

Bordetella pertussis, the agent of whooping cough, secretes an adenylate cyclase toxin-hemolysin (ACT, AC-Hly or CyaA) which paralyzes bactericidal activities of host phagocytes and is able to penetrate and intoxicate a variety of other cells [1,2]. ACT is a 1706-residue-long bifunctional protein and consists of an amino-terminal adenylate cyclase (AC) domain, comprising about 400 N-terminal residues and of an RTX (*R*epeat in *To*X*i*n) cytolysin moiety of about 1306 residues [3,4]. The RTX moiety inserts into cellular membranes and mediates translocation of the AC domain into cytosol, where it binds calmodulin and catalyzes conversion of ATP to cAMP, thereby subverting cellular signaling [5,6]. In parallel, the RTX moiety can form small cation-selective membrane channels that allow entry of calcium ions and can cause colloid-osmotic cell lysis, such as hemolysis of erythrocytes [5,7].

The RTX cytolysin moiety consists itself of a channel-forming domain (residues 500 and 700), an acylated domain (residues 800 to 1000) and of the characteristic glycine- and aspartate-rich RTX domain, which contains the nonapeptide repeats of a consensus sequence (L/I/F/)-X-G-G-X-G-X-D [8]. Binding of calcium to these repeats induces conformational changes in the toxin molecule and promotes ACT penetration into cells [9,10]. Like other RTX cytolysins, also ACT is synthesized as a protoxin (proACT). It gains the

Abbreviations: AC, adenylate cyclase; ACT, acylated adenylate cyclase toxin; CyaC, ACT-activating protein; DMPC, dimyristoyl phosphatidylcholine; DPPC, dipalmitoyl phosphatidylcholine; FITC, fluorescein isothiocyanate; LUV, large unilamellar vesicle; proACT, non-acylated adenylate cyclase toxin; RTX, *R*epeat in *To*X*i*n; SPC, soybean phosphatidylcholine

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capacity to cross cellular membranes upon a posttranslational palmitoylation of the ε-amino group of the lysine 983, which is catalyzed by a dedicated protein acyltransferase, CyaC [11,12].

Lally et al. [13] have shown that the RTX α-hemolysin of *E. coli* uses the beta 2 integrin CD11a/CD18 as receptor on leukocytes. Recently, an $\alpha_M\beta_2$ integrin (CD11b/CD18) was, indeed, identified as ACT receptor on myeloid cells [14]. This goes well with the known cytotoxic activity of ACT that ablates bactericidal functions and induces apoptosis of host phagocytes [2,15,16]. It is noteworthy, however, that ACT can also penetrate and intoxicate a variety of cells lacking CD11b [1]. For a related RTX protein, HlyA, a glycophorin was recently shown to serve as receptor on erythrocytes [17]. While macropinocytosis of ACT was recently reported to contribute to cytotoxic activity of ACT on macrophages [18], ACT appears to be a unique example of an enzymatically active toxin capable of direct translocation across the cytoplasmic membrane of cells lacking membrane trafficking mechanisms, such as mammalian erythrocytes [5,19,20]. Translocation across, but not the mere insertion of ACT into membranes, appears to require membrane potential [21]. Indirect evidence suggests that formation of ACT channels involves oligomerization of membrane-embedded ACT monomers [20,22–24] and the inner diameter of the channels, both in erythrocyte and artificial planar lipid bilayer membranes, was estimated to be between 0.6 and 0.8 nm [7,25]. Recently, a predicted transmembrane α-helix between residues 502 and 522 of ACT was shown to play a critical role in both the translocation of AC across membrane and modulation of the channel-forming activity of the toxin [24]. A helix-breaking E509P substitution within this segment selectively abolished the capacity of ACT to deliver the AC domain into erythrocytes. Moreover, while also abolishing AC domain translocation, the charge-reversing lysine substitutions of glutamates Glu-509 and Glu-516 within this putative α-helix strongly enhanced the specific hemolytic activity of ACT by upmodulating its capacity to form channels and by importantly reducing their cation-selectivity [24]. These recent results raised the hypothesis that ACT can insert into membranes in two different conformational states, one being a 'translocation precursor' allowing AC domain delivery into cells and one being a 'channel precursor' whose insertion into membranes accounts for potassium efflux from cells and eventually yields formation of oligomeric membrane channels [25].

Despite the recently achieved progress, the mechanism of membrane penetration of ACT remains poorly understood. Its analysis would greatly benefit from use of a chemically defined membrane model free of unrelated proteins that would allow a detailed analysis of lipid membrane interactions, oligomer stoichiometry and topology of membrane segments of ACT. In search for such a surrogate model, we examined here the interactions ACT with artificial lipid bilayers of liposomes.

2. Materials and methods

2.1. Chemicals

Fluorescein isothiocyanate (FITC), FITC-dextran (M_r approximately 4400), raffinose, soybean phosphatidylcholine (SPC type II), egg yolk phosphatidylcholine type XIIIE, phosphatidylserine type III, SPC type IIIS, DMPC, DPPC, trypsin and Trypsin inhibitor were purchased from Sigma. DEAE sepharose, phenyl sepharose, calmodulin-agarose, Sephadex G25 and proteinase K were from Amersham Biosciences.

2.2. Production and purification of ACT

The acylated adenylate cyclase toxin (ACT) and the non-acylated toxin (proACT) were produced in the presence, or in the absence, of the activating protein acyltransferase CyaC in *E. coli* strain XL1-Blue (Stratagene) transformed by the appropriate expression plasmids pT7CACT1 or pACT7, respectively [26,27]. The proteins were further purified by ion-exchange chromatography on DEAE Sepharose and Phenyl Sepharose [28] and stored frozen in 50 mM Tris–HCl pH 8.0, 8M urea and 2 mM EDTA (column elution buffer). Construction and purification of ACT variants carrying the substitutions E509K, E516K and E509K + E516K, respectively, were previously described [24]. Truncated ACT variants, ΔCla (pCACTΔ828–887), ΔH (pCACTΔ385–828), ΔHR1 (pCACTΔ385–1006), ΔHR2 (pCACTΔ385–1489), ΔR (pCACTΔ1009–1489), ΔC217 (pCACTΔC217), ΔAC (pCACTΔ1–373) and Cter 700 (pACTΔ1–1008), respectively, were produced and purified as previously described [10,29,30]. Protein ΔC843 (pACTΔ864–1706) was constructed by inserting a TAA stop codon in frame at position 864 of the *cyaA* gene. ΔC699 (pCACTΔC699) was constructed in the same way as the ACT1007 described earlier [10], while the *Xho*I–*Sca*I fragment was replaced in pCACT3, instead of pACT7. The integrity of all proteins was systematically controlled by SDS-PAGE and the degree of purification of all of them exceeded 90% homogeneity (data not shown).

2.3. ACT binding to liposomes

Large unilamellar vesicles (LUVs) of mean size of 400 nm were prepared by extrusion of multilamellar hand-shaken liposome vesicles in 50 mM Tris–HCl buffer, pH 8.0, with 150 mM NaCl, using the LiposoFast Basic apparatus (Avestin, Canada) with a polycarbonate membrane of 400-nm pore diameter (Avestin), as previously described [31].

Binding of ACT to liposomes could be assessed and unambiguously quantified without the need for radiolabelling of the toxin thanks to the very high specific AC enzyme activity of 400 μmol of cAMP formed per milligram of ACT protein per minute under the used assay conditions [32]. This allowed detection of the liposome-bound toxin by

assaying for the AC-catalyzed formation of radioactively labeled [^{32}P]-cAMP and tracing of the toxin down to concentrations of 0.1 ng per milliliter and per milligram of lipid (0.56 pM ACT). The proACT, ACT and truncated ACT proteins were diluted from 50 to 100 times concentrated stocks to a final concentration of 56 nM within 1 ml of liposome suspensions (LUV 400 nm) containing 1 mg of lipid in 50 mM Tris–HCl pH 8.0, 150 mM NaCl and containing either 2 mM $CaCl_2$ or 2 mM EDTA. After 60 min of incubation at 37 °C, the liposomes were washed twice in 50 mM Tris–HCl pH 8.0, 150 mM NaCl and once in 0.1 M Na_2CO_3 pH 10.5. For each washing step, the liposome suspensions were pelleted at 51 000 × *g* for 30 min at 4 °C, resuspended in the indicated buffers and transferred to fresh polypropylene centrifugation tubes in order to avoid any carry-over of ACT bound to tube walls instead of liposomes. Potential sedimentation and carry-over of aggregated ACT proteins was controlled by omitting the liposomes and it was found to be nil. For determination of the AC activity of ACT bound per milligram of lipid, the liposomes were solubilized prior to AC assay in 0.1% Triton X-100, 50 mM Tris–HCl buffer, pH 8.0, 0.2 mM $CaCl_2$. The activities of intact ACT associated in the presence of 2 mM calcium ions with 1 mg of liposome lipid were taken as the respective 100% binding activity values.

2.4. ACT binding to erythrocytes and hemolysis assay

The proACT, ACT and truncated ACT proteins were diluted from 50 to 100 times concentrated stocks to a final concentration of 56 nM within 1 ml of washed sheep erythrocyte suspensions (5×10^8 cells/ml) in 20 mM Tris pH 8.0, 150 mM NaCl (TN buffer) and 2 mM $CaCl_2$ (TNC buffer) or 2 mM EDTA (TNE buffer), respectively. After 30 min of incubation at 37 °C, the unbound ACT was removed by two washes of the cells in TN buffer at 6000 × *g* at 4 °C for 2 min, using fresh tubes at each step to avoid ACT carry over on tube walls. Remaining ACT that was not integrated into erythrocyte membrane was stripped off by a third wash in 0.1 M Na_2CO_3 (pH 10.5) and the cells were than lyzed in 50 mM Tris–HCl buffer, pH 8.0, 0.2 mM $CaCl_2$ containing 0.1% Triton X-100 for determination of cell-bound AC activity. The activities of intact ACT in the presence of 2 mM calcium ions were taken as the respective 100% activity values.

To determine the effect of protease pretreatment of erythrocytes on ACT binding and hemolysis of erythrocytes, the washed erythrocytes were incubated with 50 μg/ml of trypsin or 2 μg/ml proteinase K (sublytic concentrations), for 60 min at 37 °C. Proteases were removed by three washes of cells in TN buffer with 10 μg/ml of trypsin inhibitor (where applicable) and adjusted to 5×10^8 cells/ml in TNC buffer before use for ACT binding assays.

Hemolytic activity of ACT on protease-treated and control sheep erythrocytes was determined as described earlier [29] by photometric determination of the amount of hemoglobin released from 5×10^8 cells after 5 h of incubation with the indicated toxin concentration in 1 ml TNC at 37 °C.

2.5. Preparation of liposomes loaded by FITC

Multilamellar hand-shaken liposome vesicles were prepared in 50 mM Tris–HCl buffer, pH 8.0, with 150 mM NaCl supplemented with 4.4 mM FITC, or FITC-dextran. Large unilamellar/oligolamellar vesicles of mean size of 400 nm (LUV400) were prepared as previously described [31], by extrusion of hand-shaken liposome vesicles with the LiposoFast Basic apparatus (Avestin) equipped with a polycarbonate membrane of 400 nm pore diameter (Avestin). The non-encapsulated probe was removed by gel filtration on Sephadex G25 columns (11 × 1.5 cm) operated in 50 mM Tris–HCl buffer, pH 8.0, with 150 mM NaCl. Finally, the liposomes were concentrated by centrifugation at 51 000 × *g* at 4 °C for 30 min and resuspended in 50 mM Tris–HCl pH 8.0 and used at lipid concentration of 1 mg/ml.

2.6. Determination of FITC release

Fluorescence intensity at 520 nm was measured using continuously stirred 1 cm optical path cuvettes in a SLM 4800S fluorometer (SLM Instruments Inc., Urbana, IL) at 465 nm excitation wavelength and 37 °C. The release of the entrapped FITC from liposomes was determined as increase of FITC fluorescence intensity due to relief of self-quenching of the probe upon dilution in the liposome bathing buffer. The half-time of liposome lysis was defined as the time needed to achieve the half-maximal increase in FITC fluorescence intensity. The value for maximal (100%) vesicle leakage was determined upon liposome solubilization by 0.1% (v/v) Triton X-100. The resulting fluorescence intensities were corrected for photobleaching and basal increase of fluorescence intensity caused by spontaneous leakage of liposomes in the absence of toxin. This was typically between 8% and 10% per hour for LUVs and between 1% and 2% per hour for multilamellar vesicles.

2.7. Determination of AC activity

AC activities were measured as previously described [32]. One unit (U) of AC activity corresponds to 1 μmol of cAMP formed per minute in the presence of 1 μM calmodulin at 30 °C, pH 8.0.

3. Results

3.1. ACT binds to a protease-resistant receptor on erythrocytes

The $\alpha_M\beta_2$ (CD11b/CD18) integrin was recently identified as ACT receptor on myeloid cells [14]. ACT was, however, previously shown to penetrate also a number of

cells lacking this receptor, including human and sheep erythrocytes. On these cells, the highly trypsin-sensitive glycophorin was recently shown to serve as an alternate receptor for the related RTX protein, HlyA [17]. We, therefore, addressed the question whether ACT also binds to a protease-sensitive receptor on erythrocytes.

Direct assessment of ACT binding to cells was possible without the need for radiolabelling of the toxin, since the high specific enzymatic activity allowed to trace the toxin down to 0.56 pM concentrations (0.1 ng ACT per milliliter of cell suspension). As shown in Fig. 1, when erythrocyte surface proteins were digested at high concentrations of trypsin (50 μg/ml), or of proteinase K (2 μg/ml), no decrease of ACT binding or hemolytic activity was observed, as compared to untreated cells. In contrast, the hemolytic activity of ACT was reproducibly even higher on protease-treated than on control erythrocytes. This suggested that unlike HlyA, ACT does not bind to the trypsin-sensitive glycophorin and that the ACT receptor on erythrocytes was highly protease-resistant and it could be a non-proteinaceous species, such as a lipid or glycolipid. This would go well with previous observations that binding of ACT to erythrocytes is essentially unsaturable and could not be competitively inhibited by large excess of various ACT deletion mutants, suggesting that the toxin receptor on erythrocytes could be a rather abundant molecule [29].

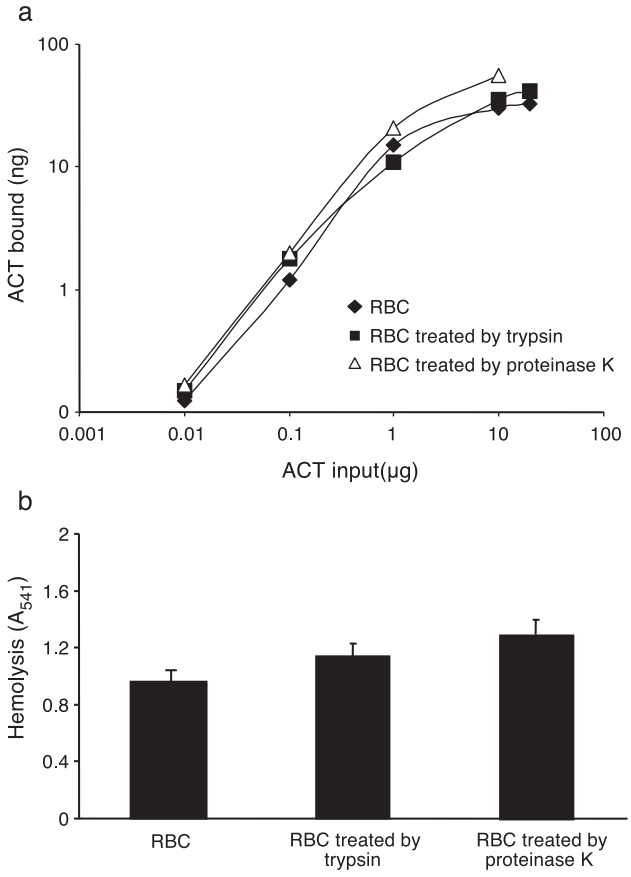

Fig. 1. Protease pretreatment of erythrocytes does not affect ACT binding and hemolytic activity. Washed sheep erythrocytes (5×10^8/ml) in TN buffer (10 mM Tris–HCl, 150 mM NaCl pH 8.0) were incubated with trypsin (50 μg/ml), or proteinase K (2 μg/ml) at 37 °C for 60 min to digest the erythrocyte surface proteins, whereupon cells were repeatedly washed in the presence of protease inhibitors. (a) Binding of ACT to protease-treated and control erythrocytes (RBC) was assessed as described in Materials and methods. A representative figure is shown for one out of three independent determinations performed in duplicates and differing by less than 15%. (b) Hemolytic activity of 1 μg/ml of ACT on 5×10^8 control and protease-treated erythrocytes (RBC) was assessed as the amount of hemoglobin (A_{541}) released after 300 min of incubation of the toxin with cells at 37 °C in TNC.

3.2. ACT inserts into the lipid bilayer of LUVs made of pure phospholipids

Gordon et al. [33] have previously shown that ACT can bind and disrupt liposomes containing phospholipids, cholesterol and sphingolipids. This also suggested that membrane interactions of ACT could be analyzed in a chemically defined lipid vesicle system in the absence of other proteins. We therefore examined in detail the interaction of ACT with large unilamellar liposomes prepared by 20 times repeated extrusion of phospholipid suspensions through filters with a cut-off limit of 400 nm (LUV 400 nm). As shown in Fig. 2a, the amount of ACT bound to 1 mg of liposomes was comparable to that of binding to 5×10^8 sheep erythrocytes, even when liposomes were made of highly purified phospolipids, such as soybean or egg yolk phosphatidylcholine, phosphatidylserine, or a DMPC/DPPC (1:1) mixture, respectively. The bound toxin could not be removed by repeated washing with buffer and subsequent stripping of liposomes with 0.1 M sodium carbonate at pH 10.5, which is an established procedure for removal of peripheral membrane proteins [34]. This suggested that ACT was able to insert into the lipid bilayer of the liposome membrane in the absence of any receptor protein and membrane potential.

As further shown in Fig. 2a, the efficiency of ACT binding was, however, greatly enhanced by lowering the homogeneity of the lipid preparation. A several-fold higher yield of bound toxin was observed with liposomes made of soybean lecithin type IIS (Sigma), which consists of only about 20% of phosphatidylcholine and the rest is a mixture of other plant lipids. However, given the about 10 times smaller total surface of 5×10^8 sheep erythrocytes, as compared to 1 mg of 400 nm large asolectin LUVs ($\sim 5 \times 10^{12}$ lipid vesicles), about three times more ACT bound per surface unit to the erythrocyte membranes than to asolectin LUVs. Since the soybean lecithin liposomes exhibited the highest binding of ACT, these were used for further analyzes.

3.3. Free calcium, but not fatty-acyl modification of the toxin, is required for binding and disruption of liposomes by ACT

Penetration of ACT across membranes of target cells depends on the posttranslational palmitoylation of its

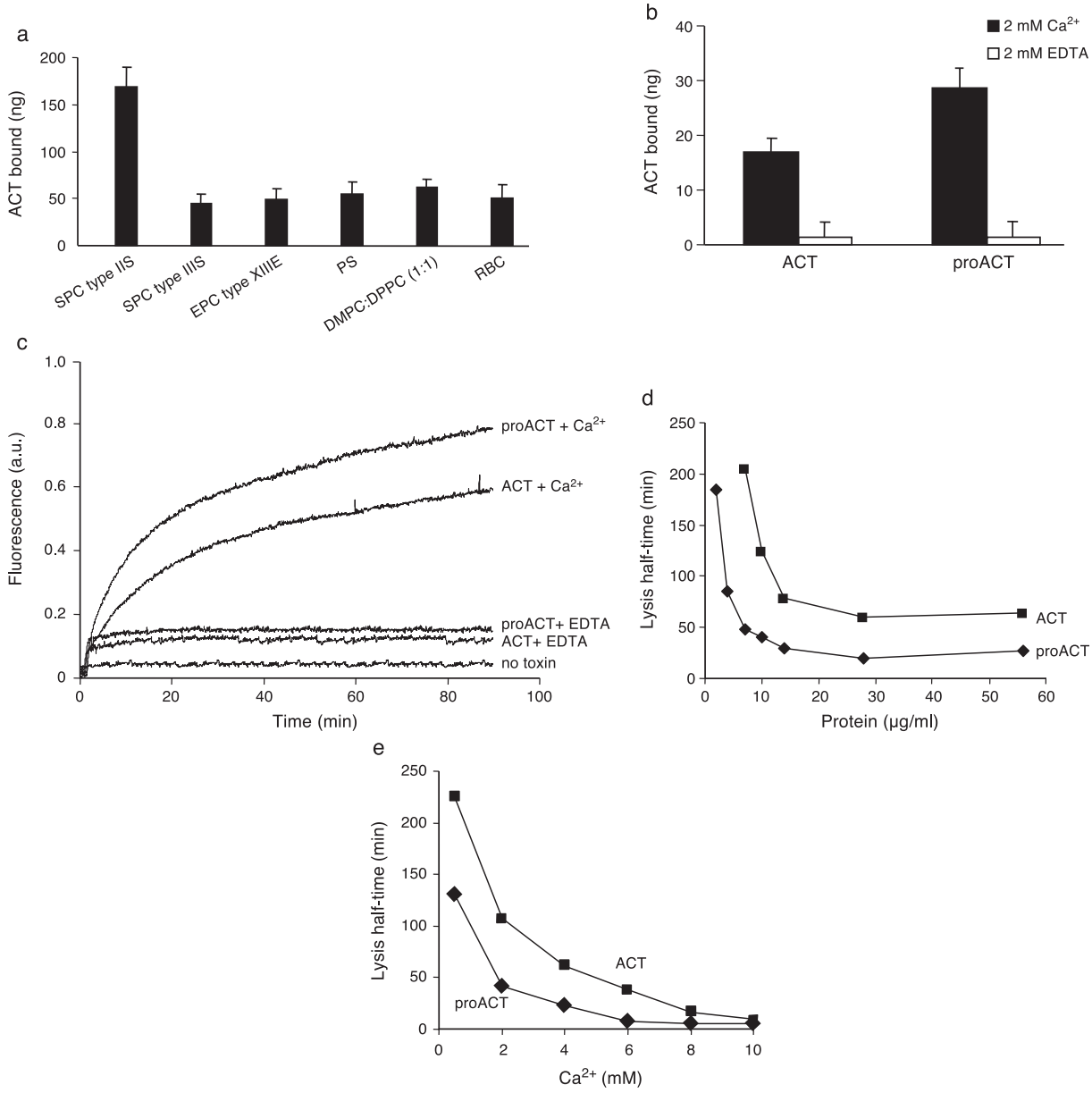

Fig. 2. ACT binds and disrupts large unilamellar liposomes of various lipid compositions in a calcium-dependent and fatty-acylation independent manner. (a) Binding of ACT to LUV400. Unilamellar/oligolamellar liposomes were prepared by 20 times repeated extrusion of asolectin, or of highly purified lipid suspensions through filters with a 400 nm cut-off pore size [31]. Ten micrograms of ACT was incubated with liposomes of various composition in 1 ml of buffer and toxin binding per milligram of lipid is shown. For comparison, binding of ACT per 5×10^8 washed sheep erythrocytes (RBC) was determined. (b) ACT binding to asolectin LUV400 requires the presence of calcium ions. Purified non-acylated proACT and acylated mature ACT were tested at 1 μg/ml for binding to soybean asolectin LUV400. Liposomes were prepared and washed in TN buffer and upon addition of either 2 mM calcium ions or 2mM EDTA, respectively, ACT binding per milligram of lipid was assessed after repeated washing and 0.1 M carbonate stripping at pH 10.5, as described under Materials and methods. (c) ACT disrupts LUV400 liposomes. Purified non-acylated proACT and ACT were tested for disruption of asolectin LUVs (1 mg/ml) with entrapped FITC probe in TNC buffer. Kinetics of FITC release induced by equal concentrations of ACT or proACT (160 nM) was followed at 37 °C in the presence of 2 mM calcium ions or 2 mM EDTA, respectively. For the negative control (no toxin), a corresponding volume of 8 M urea in TNC was added. ACT and proACT were added at a time point that corresponds to 1 min in the given recordings. Spontaneous leakage of FITC from LUVs over the 100 min of incubation was about 0.06 a.u. on a scale from 0.0 to 1.0 a.u., the latter corresponded to total dequenching of FITC fluorescence caused by detergent lysis of LUVs with 0.1% Triton X-100. The figure shows a representative set of lysis curves from one out of four independent measurements. (d) proACT disrupts MLVs faster than ACT. The dependencies of half-times of FITC probe leakage from multilamellar liposomes on ACT and proACT concentrations were determined in TNC buffer with 4 mM calcium concentration. Three independent determinations for each protein and condition were performed and representative data from one set of experiments are shown. (e) Calcium requirements of ACT and proACT for liposome disruption are similar. FITC-loaded MLVs were incubated with 160 nM proACT and ACT in TNC buffer. The probe leakage half-time corresponded to the time at which FITC fluorescence reached 0.5 a.u., e.g. 50% of total FITC dequenching caused by detergent lysis of MLVs with 0.1% Triton X-100.

lysine 983 residue and on the presence of higher than 0.1 mM free calcium ion concentrations [9,12]. As shown in Fig. 2b, omission of calcium in the liposome bathing buffer and chelation of residual traces of calcium ions by 2 mM EDTA resulted in 10-fold drop of liposome-binding capacity of ACT. Hence, in this respect, the binding of ACT to liposomes resembled toxin interaction with cells. It should be noted that in this binding experiment the toxin concentration was lowered to 1 μg/ml (Fig. 2b), in order to control that formation of toxin aggregates and their unspecific interaction with liposomes did not account for the observed ACT binding to liposomes. Moreover, the bound toxin could not be stripped off from the liposome membranes by washing in 0.1 M sodium carbonate at pH 10.5, suggesting that both ACT and proACT were inserted into liposome membranes. Surprisingly, the non-acylated proACT exhibited an about two times higher capacity to bind liposomes than the acylated mature ACT, as further shown in Fig. 2b. Moreover, both proACT and ACT induced leakage of the 398-Da FITC probe from liposomes, as shown in Fig. 2c and d. Correlating with the enhanced binding, also the kinetics of liposome disruption induced by proACT was importantly faster than that of ACT-mediated lysis. At equal protein concentrations, the proACT induced liposome lysis with two to four times shorter half-times than mature ACT (Fig. 2d). The liposome-disrupting activity of both proteins exhibited, however, a similar calcium dependency, as further shown in Fig. 2e. Altogether, these results show that the fatty-acyl modification of ACT was by itself not required for insertion of ACT into the lipid bilayer of liposome membrane and may play another role in toxin activity.

3.4. Membrane activity of ACT leads to formation of large lesions in liposome membranes

Earlier osmotic protection experiments on erythrocytes and single-channel measurements on planar lipid bilayers with applied voltage showed that ACT forms small cation-selective membrane channels with an inner diameter between 0.6 and 0.8 nm. The ACT channels appear too small already for diffusion of monosaccharides of molecular mass above 100 Da [7,25] and would not allow permeation of the 398 Da FITC probe. Indeed, as shown in Fig. 3, a quite similar kinetics was observed for ACT-mediated release of entrapped FITC and of the 10 times larger FITC-dextran conjugate probe (M_r approximately 4400) from liposomes. This strongly suggests that the membrane activity of ACT (and proACT) resulted in formation of large membrane-disrupting lesions allowing release of the fluorescent probes. In this respect, the activity of ACT on liposomes mimicked its lytic activity on erythrocytes, where hemoglobin is released upon erythrocyte disruption by colloid-osmotic lysis resulting from the membrane-permeabilizing activity of ACT channels.

3.5. Structure–function relationships determining ACT penetration of cellular membranes are partially reproduced in the liposome model

It was important to analyze how well are the structure–function relationships underlying toxin activities in cellular membranes reproduced in the surrogate membrane model. Therefore, interaction with liposomes was examined for a set of ACT variants with deletions of individual

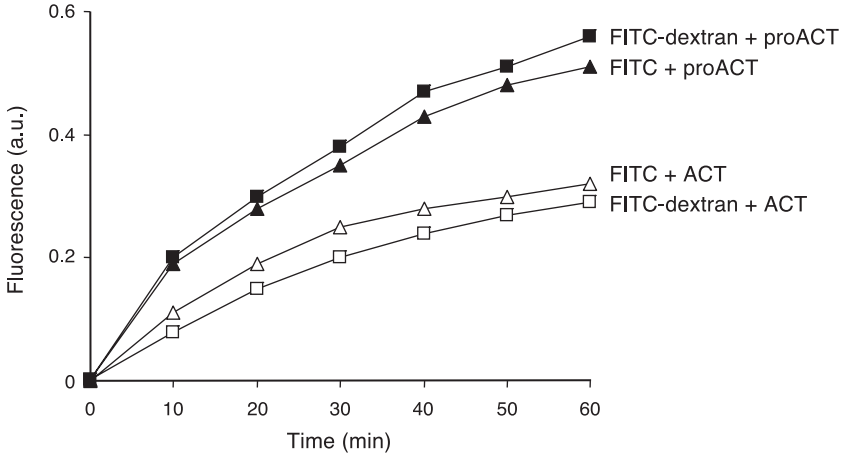

Fig. 3. Kinetics of toxin-mediated release of entrapped FITC and FITC-dextran from large unilamellar liposomes are similar. ACT- and proACT-induced kinetics of release of FITC (398 Da) and/or FITC-dextran (~ 4400 Da) from soybean lecithin LUVs was compared in the presence of 4 mM calcium ions. FITC and FITC-dextran-probe release from LUVs was induced by 160 nM ACT or proACT and was measured as increase of fluorescence intensity at 520 nm due to dilution of the released probe in the bathing buffer and the resulting relief of probe self-quenching. 1.0 a.u. corresponds to full dequenching of FITC fluorescence upon detergent lysis of LUVs with 0.1% Triton X-100.

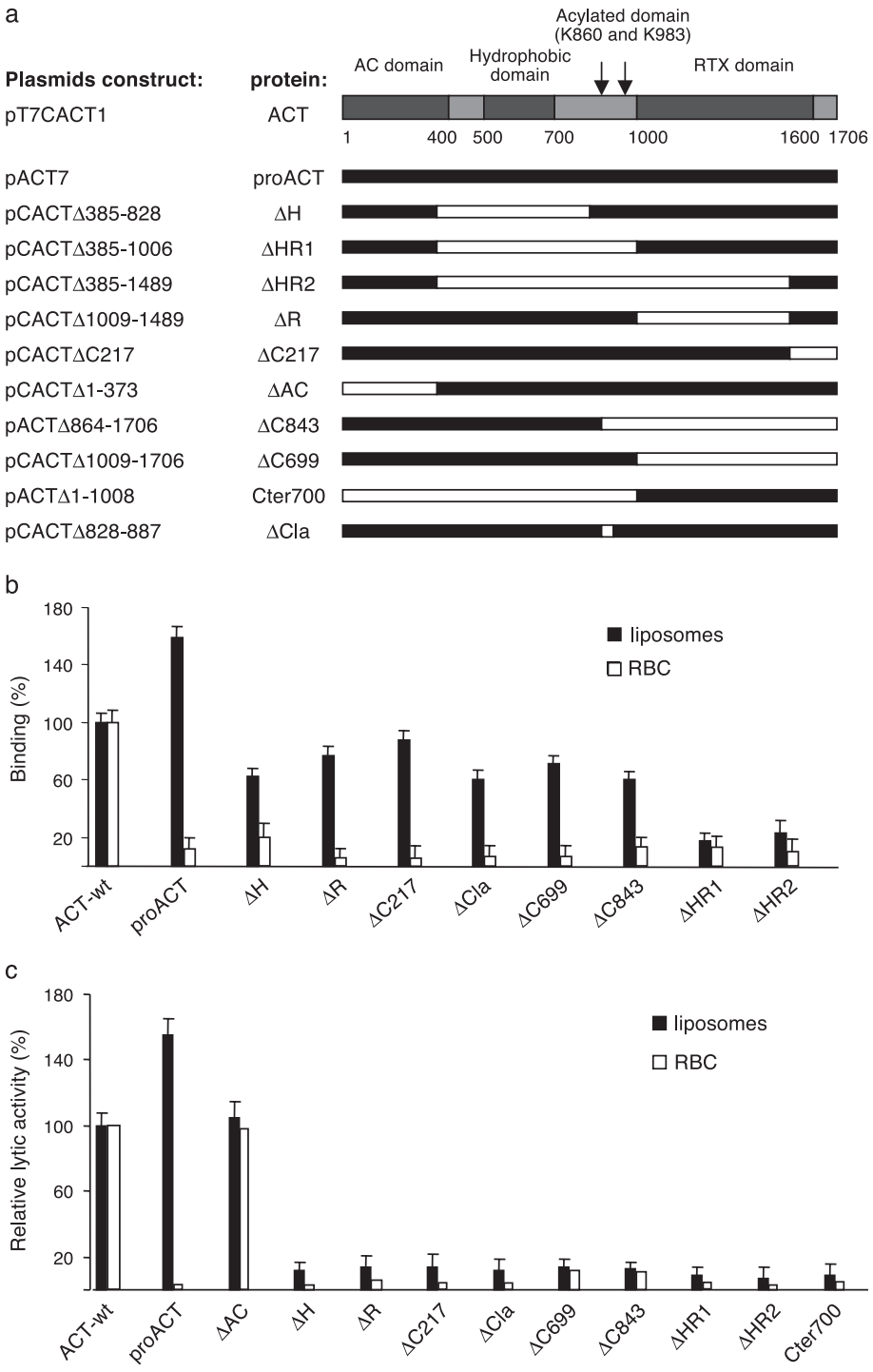

Fig. 4. Differences in structural requirements for ACT binding and lysis of erythrocytes and large unilamellar liposomes. (a) Schematic depiction of the various truncated ACT variants used in this study. Deleted portions are indicated by the open bars. In the names of the plasmids the symbol △ is followed by the numbers of the first and last codons of the deleted parts of the ACT reading frame. In the names of the respective proteins, the numbers that follow the symbol ΔC represent the number of missing C-terminal residues. (b) Deletions of individual ACT domains differently affect ACT binding to liposomes and erythrocytes, while (c) ablating both liposome disrupting and hemolytic activities of ACT. The binding capacity and lytic activities of various truncated ACT variants were determined at 56 nM concentrations of the respective protein incubated with either 1 mg of asolectin liposomes or with 5×10^8 washed sheep erythrocytes (RBC) in 1 ml of TNC at 37 °C. The activities of 56 nM intact ACT (ACT-wt) in TNC at 2 mM calcium concentration were taken as the respective 100% activity values. Prior to testing the membrane activities, concentrations of all the ACT-derived proteins were equalized on a molar basis, by using the known predicted molecular masses of the various truncated proteins for calculations. The 100% ACT-wt activity corresponded on average to 170 ng of toxin bound per milligram of lipid of LUVs resuspended in 1 ml of buffer, or to 56 ng of toxin bound per 5×10^8 erythrocytes, respectively. These levels of ACT binding caused half-maximal toxin-mediated lysis of RBC in 5 h (hemoglobin release from 5×10^8 erythrocytes yielding an A_{541} of 1.5) and about 30% lysis of LUV (0.3 fluorescence a.u.) in 1 h, respectively. The given activities represent the average value from three experiments performed in duplicates ($n = 6$).

domains and/or carrying substitutions of key residues that were previously shown to importantly affect formation of ACT channels. As shown in Fig. 4, ACT constructs with individual deletions of the repeat domain, the acylated domain and/or of the channel-forming domain, respectively, were all inactive in respect to binding and lysis of sheep erythrocytes. However, deletion of none of these individual domains caused loss of liposome insertion by itself and all such constructs exhibited over 50% capacity to insert into liposomes and could not be washed out from the membranes by stripping with sodium carbonate. Only upon a combined deletion encompassing both the channel-forming and the acylated domains (residues 385 to 1008), the ΔHR1 protein was unable to penetrate liposomes. This suggests that in parallel to the hydrophobic channel domain that could be expected to insert into the lipid bilayer, also the acylated domain could be inserting into liposome membrane, and membrane insertion of each one of these domains was sufficient for tight association of the protein with liposomes. The structural constraints on insertion of ACT into liposome membranes hence appear to be much less stringent than for insertion into erythrocyte membrane.

However, already the deletion of residues 828 to 887 (ΔCla), as well as all other examined deletions in the hemolysin moiety of ACT, ablated the capacity of the toxin to disrupt liposomes (Fig. 4c). This shows that liposome disruption by ACT was not due to a nonspecific 'detergent-like' activity of ACT, or to some artifactual interaction of toxin aggregates with liposomes. It is very unlikely that such unspecific membrane activity would be lost upon deletion of the various and often rather small portions of the toxin molecule. On the contrary, the specific liposome-disrupting activity depended on the structural integrity of the entire hemolysin portion of ACT. This corresponded well with the structural constraints on hemolytic activity of ACT on eryhrocytes [29].

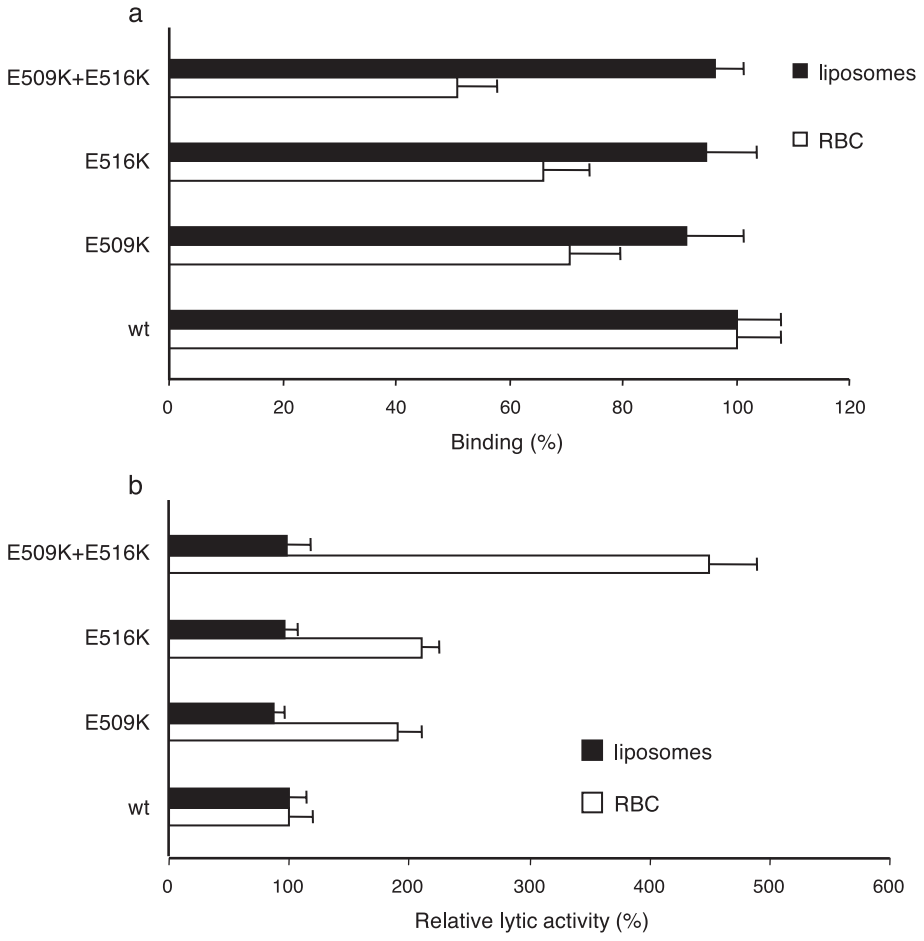

Fig. 5. Point substitutions enhancing the channel-forming and hemolytic activities of ACT do not alter its capacity to bind and disrupt liposomes. The ACT variants carrying the substitutions of glutamates E509K, E516K and E509K + E516K at positions 509 and 516, respectively, were prepared as described previously [24]. ACT binding to liposomes and erythrocytes (a) and lytic activity on liposomes (b) were compared and assessed as described in detail in the legend to Fig. 4. The relative hemolytic activity of ACT mutants (b) was scored already after 3 h of incubation with the toxins because of the much faster lysis kinetics induced by the mutant ACT. Average values of data obtained in three independent determinations performed in duplicates are given ($n = 6$).

3.6. Point substitutions enhancing channel-forming and hemolytic activity of ACT do not affect its binding and lytic activities on liposomes

To further investigate how truly does the disruption of liposomes by ACT reproduce the mechanism underlying the process of erythrocyte lysis, we examined the liposome binding and lytic activities of the ACT variants carrying single and double lysine substitutions of the glutamate residues 509 and 516, respectively. When combined, these substitutions were previously shown to enhance the specific hemolytic activity of ACT by almost an order of magnitude and to cause a strong decrease of the cation selectivity of ACT channels in parallel to the strong increase of the specific frequency of ACT channel formation in artificial planar lipid bilayers under applied voltage [24]. However, neither individually, nor in combination, did these glutamate substitutions exhibit any effect on binding and specific liposome disrupting activity of ACT (Fig. 5). These results shows that the mechanism of action and/or topology of ACT in liposomal and cellular membranes may differ.

4. Discussion

Earlier studies showed that analysis of membrane activities of ACT could be performed in chemically defined lipid membranes, since ACT was able to form distinct small cation-selective and voltage-dependent channels in planar lipid bilayers made of soybean asolectine, or pure lipids [7]. Properties of these channels, such as their inner diameter, were similar to the estimated properties of channels formed in the plasma membrane of sheep erythrocytes [25]. Moreover, there was an excellent correlation between the observed specific channel-forming activities of the various ACT forms in the planar lipid bilayer membranes and their hemolytic activities on sheep erythrocytes, when activities of the natural ACT isolated from *Bordetella*, that of recombinant ACT from *E. coli*, or those of various mutant ACT forms were compared [7,24]. The minute surface of planar lipid bilayer membranes and the low amounts of membrane–incorporated ACT in this model make it, however, very limiting for biochemical studies on ACT membrane topology.

This motivated us to examine the membrane interaction of ACT in detail using a liposome model. The capacity of ACT to disrupt liposomes was previously demonstrated [33] and liposome systems previously served fairly well for studies on a number of other toxins [35–37]. However, as shown here for ACT, the RTX toxins may represent a particular class of pore-forming toxins for which interactions with cellular membrane are not reproduced in liposomes truly enough. It was previously shown for the related RTX protein HlyA that the posttranslational acylation and presence of free calcium ions, which are both essential for the biological activity of HlyA on cells, were not necessary for liposome binding of HlyA [38–40]. In this respect, the calcium-dependence of ACT binding to liposomes reflects somewhat better the cellular interactions of the toxin. We further show here that like for insertion and translocation of ACT into erythrocyte membranes [29], the liposome-disrupting capacity of ACT required the structural integrity of the toxin. However, the same deletions that strongly affected binding of the toxin to erythrocytes failed to ablate binding of truncated ACT variants to liposomes. Moreover, the activity of ACT on asolectin liposomes was not at all affected by the lysine substitutions of glutamates 509 and 516, which strongly enhance the hemolytic activity and the channel-forming capacity of ACT in planar asolectin bilayers with membrane potential [24]. This points towards possible mechanistic or structural differences in toxin activities on cellular and planar membranes, as compared to liposomes.

The conserved requirement of structural integrity of ACT for its capacity to cause liposome and erythrocyte disruption suggests that at least the initial interaction of ACT with liposomal membranes may involve similar structures as the toxin interaction with planar bilayers and cellular membranes. In this respect, the results point to a central role of the segment deleted in the protein ΔCla and comprising residues 828 to 888, which appear important for the interaction of ACT with both cellular and liposome membranes. Indeed, it was previously shown that a rather conservative substitution of the lysine 860 residue (K860) within this segment by an arginine residue importantly reduced the capacity of ACT to penetrate erythrocyte membranes, independently of the acylation status of K860 [11,44]. However, the nil effect of the substitution of glutamates 509 and 516 on liposome binding and disrupting activity of ACT suggests that ACT may insert and act differently in liposome and planar bilayer membranes. Disruption of liposomes by ACT does, moreover, not appear to be a result of osmotic lysis, since it could not be inhibited by externally added raffinose (data not shown). It is conceivable that the toxin may be inserting inappropriately into liposomal membrane and instead of forming true cation-selective transmembrane channels, insertion of ACT may destabilize the membrane and result in formation of large lesions causing liposome disruption and release of the entrapped fluorescent probe. A similar mechanism was, indeed, recently suggested for liposome disruption caused by HlyA [43]. This may represent a major hurdle for accurate reproduction of ACT interactions with biological membranes in liposomes.

Surprisingly, while acylation was strictly required for ACT channel activity in planar lipid bilayers [7,20], the acylation was not required here for binding and disruption of liposomes by the protoxin. Furthermore, the non-acylated proACT exhibited even higher specific liposome binding and lytic activity than mature ACT. This shows that the fatty-acylation of ACT is not essential for the mere interaction of the toxin with the naked lipid membrane per se. The results, indeed, suggest that acylation of ACT may rather be

required for interaction of ACT with other cellular components than the cellular lipid bilayer itself. Secondly, it is possible, that the conformations of the acylated ACT and non-acylated proACT proteins may differ. The non-acylated proACT may adopt a conformation allowing it to insert more readily into the naked liposome membrane, while the acylated ACT would adopt a conformation required for a productive interaction with the glycocalyx or protein components shielding the cellular membrane. Other results suggest that acylation plays a functional role in toxin activity, such as modulating the propensity of ACT to form membrane oligomers. This hypothesis goes well with the observations that modification of ACT by fatty acyl chains of differing properties did not affect the overall capacity of ACT to penetrate cellular membranes and it affected selectively only the specific capacity of ACT to form ionic channels and lyze erythrocytes [7,11,41,42].

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