**Bordetella Adenylate Cyclase Toxin Mobilizes Its β2 Integrin Receptor into Lipid Rafts to Accomplish Translocation across Target Cell Membrane in Two Steps**

Ladislav Bumba¹, Jiri Masin¹, Radovan Fiser², Peter Sebo¹³

1 Institute of Microbiology AS CR v.v.i., Prague, Czech Republic, 2 Faculty of Science, Charles University, Prague, Czech Republic, 3 Institute of Biotechnology AS CR v.v.i., Prague, Czech Republic

Abstract

*Bordetella* adenylate cyclase toxin (CyaA) binds the αMβ2 integrin (CD11b/CD18, CR3 or Mac-1) of myeloid phagocytes and delivers into their cytosol an adenylate cyclase (AC) enzyme that converts ATP into the key signaling molecule cAMP. We show that penetration of the AC domain across cell membrane proceeds in two steps. It starts by membrane insertion of a toxin ‘translocation intermediate’, which can be ‘locked’ in the membrane by the 3D1 antibody blocking AC domain translocation. Insertion of the ‘intermediate’ permeabilizes cells for influx of extracellular calcium ions and thus activates calpain-mediated cleavage of the talin tether. Recruitment of the integrin-CyaA complex into lipid rafts follows and the cholesterol-rich lipid environment promotes translocation of the AC domain across cell membrane. AC translocation into cells was inhibited upon raft disruption by cholesterol depletion, or when CyaA mobilization into rafts was blocked by inhibition of talin processing. Furthermore, CyaA mutants unable to mobilize calcium into cells failed to relocate into lipid rafts, and failed to translocate the AC domain across cell membrane, unless rescued by Ca²⁺ influx promoted in trans by ionomycin or another CyaA protein. Hence, by mobilizing calcium ions into phagocytes, the ‘translocation intermediate’ promotes toxin piggybacking on integrin into lipid rafts and enables AC enzyme delivery into host cytosol.

Introduction

The secreted adenylate cyclase toxin-hemolysin (CyaA, ACT, or AC-Hly) plays a key role in virulence of *Bordetella*. This multifunctional protein binds the αMβ2 integrin (CD11b/CD18, CR3 or Mac-1) of myeloid phagocytes and delivers into their cytosol a calmodulin-activated adenylate cyclase enzyme that ablates bacterial capacities of phagocytes by uncontrolled conversion of cytosolic ATP to the key signaling molecule cAMP [1–5]. In parallel, the hemolysin moiety of CyaA forms oligomeric pores that permeabilize cell membrane for monovalent cations and contribute to overall cytotoxicity of CyaA towards phagocytes [6–10].

The toxin is a 1706 residues-long protein, in which a 400 N-terminal residues (Glu 509/Glu516 and Glu 570/Glu581) that were found to be essential amphipatic transmembrane segments within the pore-forming activity [19,20]. The latter consists itself of three functional domains typical for RTX hemolysins. It harbors, respectively, (i) a hydrophobic pore-forming domain, (ii) a region recognized by the protein acyltransferase CyaC, activating proCyaA by covalent post-translational palmitoylation at ε-amino groups of Lys860 and Lys983 [12,13], and (iii) an assembly of five blocks of the characteristic glycine and aspartate-rich nonapeptide RTX repeats that form numerous (~40) calcium-binding sites [14].

Since no structural information on the RTX cytolysin moiety is available, the mechanistic details of toxin translocation across the lipid bilayer of cell membrane remain poorly understood. Delivery of the AC domain into cells occurs directly across the cytoplasmic membrane, without the need for toxin endocytosis [15] and requires structural integrity of the CyaA molecule [6], unfolding of the AC domain [16] and a negative membrane potential [17]. Recently, we described that CyaA forms a calcium-conductive path in cell membrane and mediates influx of extracellular Ca²⁺ ions into cell cytosol concomitantly with translocation of the AC domain polypeptide into cells [18].

The current working model predicts that both Ca²⁺ influx and AC translocation depend on a different membrane-inserted CyaA conformer than the pore-forming activity [19,20]. The two membrane activities of CyaA, however, appear to use the same essential amphipatic transmembrane segments within the pore-forming domain (ε-helix350–352 and ε-helix565–591), employing them in an alternative and mutually exclusive way. These segments harbor two pairs of negatively charged glutamate residues (Glu350/Glu352 and Glu565/Glu591) that were found to play a central role in toxin action on cell membrane. These control, respectively, the translocation of the positively charged AC domain, the formation of oligomeric CyaA pores and the cation-selectivity of the CyaA pore. Charge-reversing, neutral or
Author Summary

The adenylate cyclase toxin (CyaA) of pathogenic Borde-
tellae eliminates the first line of host innate immune 
defense. It penetrates myeloid phagocytes, such as 
neutrophils, macrophage or dendritic cells, and subverts 
their signaling by catalyzing an extremely rapid conversion 
of intracellular ATP to the key signaling molecule cAMP. 
This efficiently inhibits the oxidative burst and comple-
ment-mediated opsonophagocytic killing of bacteria, thus 
enabling the pathogen to colonize host airways. We show 
that translocation of CyaA into phagocyte cytosol occurs 
in two steps. The toxin first binds the integrin CD11b/CD18 
and inserts into phagocyte membrane to mediate influx 
of calcium ions into cells. This promotes relocation of 
the toxin-receptor complex into specific lipid microdomains 
within cell membrane called rafts. The increased concen-
trations of cholesterol within rafts and their particular lipid 
organization then support translocation of the adenylate 
cyclase enzyme directly into the cytoplasmic compartment 
of cells. The mechanism of CyaA penetration into cells sets 
a new paradigm for membrane translocation of toxins of 
the RTX family.

CyaA Translocation across Cell Membrane

helix-breaking substitutions of these glutamates were found to shift 
the balance between AC translocating and pore-forming activities 
of CyaA on cell membrane [8,10,19,20].

The very high specific AC enzyme activity of CyaA allowed 
previously to detect its capacity to promiscuously bind and 
penetrate at reduced levels also numerous cell types lacking the 
CD11b/CD18 receptor [21,22]. This is likely due to a weak 
lectin activity of CyaA, which would enable interaction of the 
toxin with cell surface gangliosides [23] and glycoproteins [24]. 
Indeed, binding of CyaA to CD11b/CD18 was recently found 
to depend on initial interaction with the N-linked gycan 
antaena of the receptor [24], where the specificity of CyaA for 
CD11b/CD18 appears to be determined by a segment of the 
stalk domain of the CD11b subunit (Osicka et al., manuscript in 
preparation).

CD11b/CD18 belongs to the β2 subfamily of polyfunctional 
integrins playing a major role in leukocyte function. The same β2 
subunit (CD18) can, indeed, pair with four distinct α subunits to 
yield the α1β2 (CD11a/CD18, LFA-1), αMβ2 (CD11b/CD18, 
CR3, Mac1), αXβ2 (CD11c/CD18, p150/195) and α5β2 
(CD11d/CD18) receptors, respectively [25]. Among key features 
of these integrins is their capacity of bi-directional signaling, where 
the avidity and conformation of the integrins is regulated by 
intracellular signals in the ‘inside-out’ signaling mode. In turn, 
binding of ligands or counter-receptors results in ‘outside-in’ 
signaling [26]. Among other effects, the latter yields actin 
cytoskeletal rearrangements and can result in lateral segregation 
of the β2 integrins from the bulk phase of the plasma membrane 
into distinct lipid assemblies known as lipid rafts [27,28]. These 
were first detected as detergent-resistant membrane (DRM), 
characterized by insolubility in some detergents under certain 
conditions and enriched in cholesterol, sphingolipids, and 
glycosphosphatidylinositol-anchored proteins [29–32]. Besides 
playing an important role in signal transduction, receptor 
internalization, vesicular sorting or cholesterol transport [33], 
the components of lipid rafts are often exploited as specific 
receptors mediating cell entry of toxins, pathogenic bacteria, or 
viruses [34–36].

Here, we show that CyaA-mediated influx of Ca2+ ions into cells 
induces mobilization of the toxin-receptor complex into lipid rafts,
CD11b/CD18, while lacking one or more of the other CyaA activities (Table 1). As documented in Fig. 2A, the capacity of CyaA to elevate cellular cAMP concentrations was not required for mobilization of CyaA into DRM. The enzymatically-inactive CyaA-AC2 construct, unable to catalyze conversion of ATP into cAMP, was indeed accumulating in DRM with the same efficacy as the intact CyaA (fractions 3–4). Fatty-acylation of CyaA as such was also not essential for association of CyaA with DRM. As further shown in Fig. 2A, the non-acylated proCyaA was detected in DRM despite an importantly reduced capacity to associate with cells. Moreover, the pore-forming activity of CyaA was both insufficient and dispensable for mobilization of CyaA into DRM. The acylated CyaA DAC construct, lacking the entire AC domain but retaining an intact pore-forming (hemolytic) capacity, was unable to mobilize into DRM (Fig. 2A). In contrast, the CyaA-E570Q+K860R-AC construct unable to permeabilize cells to any significant extent, but exhibiting an intact capacity to translocate the AC domain across cell membrane was, indeed, recruited into DRM together with CD11b/CD18 as efficiently as intact CyaA. In turn, the CyaA-E570K+E581P double mutant that was unable to form CyaA pores, or to translocate the AC domain across membrane, and retained only the CD11b-binding capacity (Table 1), was also unable to associate with DRM.

To corroborate these observations, we used fluorescence microscopy to examine the distribution of individual CyaA proteins in cell membrane. As documented in Fig. 2B, the intact CyaA, CyaA-AC2 and CyaA-E570Q+K860R-AC proteins were found to induce formation of, and to localize within, membrane patches. Moreover, the same patches were labeled to high extent also with B subunit of cholera toxin (CtxB), which specifically binds the GM1 ganglioside accumulating in lipid rafts. Hence, the three CyaA variants capable of associating with DRM (cf. Fig. 2A) were also found to co-localize with CtxB within membrane patches. In turn, no formation of membrane patches, a diffuse distribution on cell surface, and low if any co-localization with CtxB, were observed for the CyaA DAC and CyaA-E570K+E581P constructs that were unable to associate with DRM, too.
The pattern of DRM association, processing to the 160 kDa form and co-localization of the different CyaA variants with CtxB, respectively, resembled strongly the pattern of structure-function relationships observed recently for the capacity of CyaA to promote influx of extracellular calcium ions into J774A.1 cells [18]. Indeed, as documented in Fig. 2C by measurements of intracellular calcium concentrations ([Ca^{2+}]), the CyaA, CyaA-AC− and CyaA-E570K+K860R-AC proteins (17 nM) exhibited an expected capacity to promote Ca^{2+} influx into J774A.1 cells (see [18] for details on different kinetics of Ca^{2+} entry for AC− and AC+ constructs). In contrast, the CyaA-E570K+E581P and CyaA-AC constructs, failed to mediate any increase of [Ca^{2+}], even when used at a 113 nM concentration (Fig. 2D). Collectively, hence, these results show that the capacity of different CyaA variants to associate with DRM and co-localize with CtxB within coalesced lipid rafts was mirrored by the capacity to promote Ca^{2+} influx into cells.

Membrane insertion of a ‘translocation intermediate’ allows calcium influx and CyaA association with DRM

We showed recently that CyaA-mediated influx of Ca^{2+}− into cells is independent of the AC enzyme or pore-forming (hemolytic) activities of CyaA and occurs concomitantly to translocation of the AC domain across target cell membrane [18]. It remained, however, to assess whether it was the mere insertion of a CyaA translocation precursor into cell membrane, or whether the accomplishment of translocation of the AC domain across cell membrane was required for formation of a calcium conductive path in cell membrane. Towards this aim, we used the 3D1 monoclonal antibody (MAb) that binds to the distal end of the AC domain for details on different kinetics of Ca^{2+} entry for AC− and AC+ constructs) in cell membrane. Hence, 3D1 binding uncoupled translocation of the AC domain from membrane insertion of CyaA and ‘locked’ the toxin in the conformation of a ‘translocation intermediate’ that permeabilized cells for Ca^{2+} ions and associated with DRM.

Influx of Ca^{2+} induces association of CyaA with DRM

Next, we aimed to determine whether elevation of [Ca^{2+}], as such would mobilize into DRM also the CyaA-E570K+E581P protein unable to associate with DRM on its own (cf. Fig. 2). As demonstrated in Fig. 4, upon permeabilization of cells for extracellular Ca^{2+} ions with the Ca^{2+} ionophore ionomycin (500 nM), up to 13% of the added CyaA-E570K+E581P was found associated with DRM. In contrast, no association of CyaA-E570K+E581P with DRM was observed upon treatment of cells with 1 μM thapsigargin that increases [Ca^{2+}], by triggering Ca^{2+} release from intracellular stores. This showed that entry of extracellular Ca^{2+} across the cytoplasmic membrane was required for mobilization of CyaA into DRM.

Recruitment of CyaA-CD11b/CD18 complexes into rafts depends on cleavage of cytosolic talin by the Ca^{2+}-activated protease calpain

Influx of extracellular Ca^{2+} during leukocyte activation was reported to induce mobilization of integrins in cell membrane by calpain-mediated cleavage of talin that tethers β2 integrins to actin cytoskeleton [40,41]. Therefore, we examined whether CyaA-promoted recruitment of the toxin receptor into rafts depended on talin processing. As shown in Fig. 5A, intact talin (~270 kDa) was largely predominating in lysates of cells treated with the CyaA-ΔAG or CyaA-E570K-E381P proteins that are unable to promote Ca^{2+} entry into cells. In contrast exposure of cells to the CyaA, CyaA-AG−, or CyaA-E570Q+K860R-AG− proteins, promoting

Table 1. Relative toxin activities of CyaA-derived constructs.

| Protein          | AC enzyme activitya | CD11b+ cell binding [%]b | Hemolytic activity [%]c | AC domain translocation [%]d | Calcium influx* |
|------------------|---------------------|--------------------------|------------------------|----------------------------|-----------------|
| CyaA             | +                   | 100                      | 100                    | 100                        | +++             |
| CyaA-AC−         | −                   | N.D. (100%)              | 96±12                  | N.D. (100%)                | +++             |
| proCyaA          | +                   | 23±8                     | <1                    | 3±1                        | +               |
| CyaA-E570K+E581P | +                   | 91±15                    | <1                    | 1.8±0.5                    | –               |
| CyaA-ΔAC         | −                   | N.D. (100%)              | 109±15                | –                          | –               |
| CyaA-E570Q+K860R-AC− | −             | N.D. (100%)              | <1                    | N.D. (100%)                | +++             |

aCapacity to catalyze conversion of ATP to cAMP.
bAC enzyme activity associated with J774A.1 cells (10^6/ml) upon incubation with 6 nM protein for 30 min at 4°C. Relative activity of intact CyaA was taken as 100%.
cDetermined as the amount of hemoglobin (A562 nm) released from washed sheep erythrocytes (5.10^7/ml) by 5 μg/ml of protein at 37°C.
dAmounts of intracellular calcium per 10^7 J774A.1 cells incubated with indicated proteins for 30 min at 37°C.

*The number of plus signs reflects the relative ability of CyaA proteins to increase [Ca^{2+}]i levels (c.f. Fig. 2).

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As shown in Fig. 3C, however, despite arrested AC domain translocation, the CyaA-3D1 complex promoted elevation of [Ca^{2+}]i in cells with kinetics resembling the Ca^{2+} influx produced by CyaA-AC− (cf. Fig. 2C). Hence, 3D1 binding uncoupled translocation of the AC domain from membrane insertion of CyaA and ‘locked’ the toxin in the conformation of a ‘translocation intermediate’ that permeabilized cells for Ca^{2+} ions and associated with DRM.

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Recruitment of CyaA-CD11b/CD18 complexes into rafts depends on cleavage of cytosolic talin by the Ca^{2+}-activated protease calpain

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Figure 2. Mobilization of CyaA into lipid rafts depends on CyaA-mediated Ca²⁺ influx. (A) J774A.1 cells were incubated with 1 nM CyaA-derived proteins at 37°C for 10 min, cell lysates were fractionated on sucrose gradients and proteins were detected by immunoblots as described for Fig. 1. (B) J774A.1 cells were incubated at 37°C for 10 min with 6 nM CyaA proteins labeled with Alexa Fluor 488 before 5 μg/ml of Alexa Fluor 594-labeled recombinant cholera toxin subunit B (CtxB) was added for additional 5 min. The cell-bound proteins were visualized by fluorescence microscopy and co-localization of CyaA (green) with CtxB (red) was assessed in the merged images (yellow). Representative images from two independent experiments are shown. Note that intact CyaA induced the reported cAMP-dependent ruffling of J774A.1 cells [2]. (C and D) CyaA induces increase of cytosolic calcium concentration ([Ca²⁺]ᵢ). J774A.1 cells were loaded with the Ca²⁺ probe Fura-2/AM (3 μM) at 25°C for 30 min and exposed to 17 nM (C) or 113 nM (D) CyaA proteins. Time course of Ca²⁺ entry was recorded as the ratio of fluorescence intensities (excitation at 340/380 nm, emission 505 nm), as previously described [18]. The shown curves are representative of at least three independent experiments. doi:10.1371/journal.ppat.1000901.g002
influx of Ca\(^{2+}\) into cells, increased about seven-fold the detected amounts of the \(\sim 220\) kDa C-terminal fragment of processed talin (Fig. 5A, left panel). Concomitantly, increased amounts of the 47-kDa N-terminal fragment of talin (talin head) were detected in cell lysates. Moreover, tightly associated talin head was found to float together with the CD11b/CD18 heterodimer in DRM (Fig. 5B) and could be co-immunoprecipitated with the integrin on beads coated with anti-CD11b antibody. The relative amounts of processed CyaA (\(\sim 160\) kDa) within individual gradient fractions were determined as in Fig. 1F. Values represent the mean \(\pm\) S.D. of three independent experiments. An asterisk indicates a statistically significant difference (*, \(p<0.05\); Student’s \(t\) test). N.D., not determined.

CyaA-mediated influx of Ca\(^{2+}\) into cells was clearly due to activation of calpain, as preincubation of cells with 100 \(\mu\)M calpeptin blocked talin cleavage in CyaA-treated cells (Fig. 5A, right panel). Remarkably, pretreatment of cells with calpeptin strongly inhibited also the association of CyaA with DRM (Fig. 5D) and decreased by at least a factor of two the capacity of cell-associated CyaA to translocate the AC enzyme into target cells (Fig. 5E). In turn, no effect of calpain inhibition was observed for CyaA-mediated Ca\(^{2+}\) influx (Fig. 5F). In line with these results, pretreatment of cells with 100 \(\mu\)M calpeptin blocked effectively also the formation of CyaA-AC\(^{2+}\) patches in cell membrane and ablated co-localization of CyaA-AC\(^{2+}\) with CtxB, as documented in Fig. 5G. It can, hence, be concluded that CyaA-mediated influx of Ca\(^{2+}\) into cells activated cleavage of talin by calpain and this was required for mobilization of CyaA-CD11b/CD18 complexes into lipid rafts.

Figure 3. Binding of 3D1 antibody uncouples AC translocation from membrane insertion and CyaA-mediated Ca\(^{2+}\) influx. (A) 17 nM CyaA was preincubated for 20 min at 37°C with 20 \(\mu\)g/ml of 3D1 MAb (CyaA+3D1) or the Tu-01 IgG1 isotype control MAb (CyaA+isotype control), before 6 nM toxin was added to J774A.1 cells. CyaA binding was determined as the amount of total cell-associated AC enzyme activity upon cells incubation with 6 nM CyaA for 10 min at 37°C in the presence or absence of the indicated antibody. AC domain translocation was assessed by determining the intracellular concentration of cAMP generated in cells in the presence or absence of the indicated antibodies, following incubation of cells with four different toxin concentrations from within the linear range of the dose-response curve (0.05, 0.1, 0.25, and 0.5 nM). The % of cAMP accumulation in cells at each toxin concentration was calculated, taking cAMP values for CyaA preincubated in buffer alone as 100%. The average of such determined % activity values is given. An asterisk indicates a statistically significant difference (*, \(p<0.01\); Student’s \(t\) test). (B) J774A.1 cells were exposed to 1 nM CyaA alone, or to 1 nM CyaA preincubated with 3D1 or IgG1 isotype MAb as above. Cell lysates were separated on sucrose density gradients and analyzed as in Fig. 1. The 3D1 and isotype IgG1 MAbs were detected with anti-mouse IgG antibody. The relative amounts of processed CyaA (\(\sim 160\) kDa) within individual gradient fractions were determined as in Fig. 1F. Values represent the mean \(\pm\) S.D. of three independent experiments. An asterisk indicates a statistically significant difference (*, \(p<0.05\); Student’s \(t\) test). N.D., not determined. (C) J774A.1 cells were loaded with Fura-2/AM as above and exposed to 17 nM CyaA alone, or to CyaA preincubated with 3D1 or an IgG1 isotype control MAb. Ca\(^{2+}\) influx was recorded as above and the shown curves are representative of three independent experiments.

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ions into cells.

promote Ca\(^{2+}\) influx into cells, as shown in Fig. 6B. In parallel, the specific capacity of CyaA to translocate the AC domain across cellular membrane was quantified as in Fig. 1 and the values represent the mean \(\pm\) S.D. of three independent experiments. An asterisk indicates a statistically significant difference (*, \(p<0.05\); Student’s \(t\) test).

In Fig. 4A, cells were incubated at 37°C for 10 min with 1 nM CyaA and CyaA-E570K+E581P, in the presence or absence of 500 nM ionomycin, or of 1 \(\mu\)M thapsigargin, respectively. Cell lysates were analyzed on sucrose density gradients as above. The blots are representative of four independent experiments. In Fig. 4B, Distribution of CyaA proteins in gradient fractions was quantified as in Fig. 1 and the values represent the mean \(\pm\) S.D. of three independent experiments. An asterisk indicates a statistically significant difference (*, \(p<0.05\); Student’s \(t\) test).

Ca\(^{2+}\) influx, lipid raft association and AC translocating activity of CyaA depend on cholesterol content of target cell membrane

To determine what role does association of CyaA with lipid rafts play in the mechanism of toxin action on cellular membrane, we analyzed the activities of CyaA on cells having the rafts disrupted by depletion of cholesterol. As shown in Table 2, the total cholesterol content of J774A.1 cells could be decreased about two-fold by cholesterol extraction with 10 mM MβCD for 30 min. While the disruption of raft structures did not impact on association of CyaA with cells (Fig. 6A and Fig. S1), the modest decrease of cellular cholesterol content yielded an about five-fold decrease of the capacity of CyaA to translocate the AC domain across cell membrane. This defect was further mirrored by decreased DRM association of CyaA in MβCD-extracted cells, as shown in Fig. 6B. In parallel, the specific capacity of CyaA to promote Ca\(^{2+}\) influx into cholesterol-depleted cells was reduced and the [Ca\(^{2+}\)] increase ensuing toxin addition was delayed by several minutes, reaching a plateau at about a half-maximal [Ca\(^{2+}\)] concentration, as compared to non-depleted cells (Fig. 6C). In line with this, the two-fold decrease of cellular cholesterol level moderately decreased also the co-localization of CyaA with CtxB in lipid rafts (Fig. 6D).

Therefore, the above described experiments were replicated on monocytic U937 histiocytic lymphoma cells (CD11b\(^{+}\)) that are defective in endogenous cholesterol synthesis. These cells can be efficiently depleted of cholesterol without losing viability, by them growing for 48 hours in media containing cholesterol-free (delipidated) serum. As shown in Table 2, such treatment reduced the cholesterol content of U937 cells almost 10-times.

As shown in Fig. 6E, a pronounced, over ten-fold decrease of specific AC translocation capacity of CyaA was observed on U937 cells grown in media with delipidated serum, as compared to CyaA activity on cells grown with standard serum. At the same time, however, the total amounts of cell-associated CyaA remained equal, irrespective of cell treatment. However, by difference to well-detectable DRM association of CyaA on cholesterol-replete U937 cells, grown with standard serum, no association of CyaA with DRM was observed in lysates of cholesterol-depleted U937 cells grown in delipidated serum, respectively (Fig. 6F).

Intriguingly, compared to the Ca\(^{2+}\) influx elicited by equal concentrations of CyaA in J774A.1 cells, about an order of magnitude lower amplitude and delayed kinetics of CyaA-mediated Ca\(^{2+}\) influx was observed for U937 cells grown in media with standard serum (cf. Fig. 6C and Fig. 6G). These cells exhibited a 3-fold lower cholesterol content than the J774A.1 cells (see Table 2), suggesting that the low cholesterol content of U937 cells might have accounted for the poor capacity of CyaA to elicit Ca\(^{2+}\) influx in these cells. Indeed, when cholesterol content of J774A.1 cells was reduced about two-fold by cholesterol extraction with 10 mM MβCD, a delayed kinetics of CyaA-induced influx of Ca\(^{2+}\) into J774A.1 cells and a two-fold lower final [Ca\(^{2+}\)], reached in 20 minutes, were also observed (cf. Fig. 6C). Similarly, a delayed influx of Ca\(^{2+}\) and a lower final level of [Ca\(^{2+}\)] was observed also upon addition of equal CyaA concentrations to U937 cells depleted of cholesterol by growth in delipidated media, as compared to U937 cells grown in standard media, as shown in Fig. 6G. At the same time, however, the respective amounts of CyaA associated per 10\(^{6}\) J774A.1 or U937 cells remained the same (\(\sim 5\) ng of CyaA bound per 10\(^{6}\) cells), irrespective of whether the cholesterol content of cells was decreased by the treatments (cf. Fig. 6A and 6E). These results, hence, strongly point towards a close relation between the overall content of cholesterol in cellular membrane and the propensity of the membrane-inserted CyaA to adopt the ‘translocation intermediate’ conformation, which would account for the Ca\(^{2+}\) conducting path across cell membrane (cf. Fig. 3 and [18]).

Finally, a correspondingly reduced CtxB binding and little if any co-localization of CtxB with CyaA were observed on cholesterol-depleted U937 cells, grown in delipidated serum, as compared to binding and some observable co-localization of CyaA with CtxB on cholesterol-replete U937 cells (Fig. 6).

Mobilization of CyaA into lipid rafts enables translocation of AC domain across membrane

In the light of the above results, we aimed to test the hypothesis that AC translocation across membrane was supported and accomplished upon recruitment of the membrane-associated toxin into the cholesterol-rich environment of lipid rafts. Therefore, we examined whether the inactive CyaA-E570K+E581P construct would gain any capacity to translocate its enzymatically active AC domain across cellular membrane upon mobilization into lipid rafts. Since this mutant is intact for receptor binding but fails to promote Ca\(^{2+}\) influx into cells, we reasoned that mobilizing Ca\(^{2+}\) ions into cells in trans, by co-incubation with a translocating CyaA-AC\(^{+}\) toxoid, might promote recruitment of CyaA-E570K+E581P mutant into rafts to some extent.

As shown in Fig. 7A, when biotinylated CyaA-E570K+E581P was added to cells alone, or when it was co-incubated with equal
amounts of the enzymatically inactive CyaA-E570K+E581P-AC\textsuperscript{2} toxoid, unable to cause calcium influx, the CyaA-E570K+E581P-biotin failed to associate with DRM. In contrast, upon co-incubation with equal amounts of the translocating CyaA-AC\textsuperscript{2} toxoid [1:1], a significant fraction of CyaA-E570K+E581P-biotin associated with DRM. Moreover, as shown in Fig. 7B, this
Table 2. Cholesterol content in J774 and U937 cells.

| cells                      | ng of cholesterol per 10^6 cells |
|----------------------------|----------------------------------|
|                            | untreated | extracted  |
| J774A.1                   | 290±35    | 140±43    |
| U937 - normal serum       | 89±12     | 47±8      |
| U937 - delipidated serum  | 8±3       | N.D.      |

Cholesterol was extracted from cells using 10 mM M(II)CD at 37°C for 30 min. U937 cells deficient in endogenous cholesterol synthesis were grown in RPMI medium supplemented with 10% normal fetal calf serum or with 10% lipoprotein-deficient serum from fetal calf (delipidated serum) for 48 h. Cholesterol content was determined using the Amplex Red Cholesterol Assay Kit (Molecular Probes). Values represent the mean ± S.D. obtained from three independent experiments. N.D., not detectable.

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We show here that membrane translocation of the adenylate cyclase domain of CyaA occurs by a two step mechanism and involves toxin piggybacking on the αMβ2 integrin for relocation into lipid rafts. The present results allow us to propose a new model of CyaA mechanism of action, as summarized in Fig. 8. Upon initial binding of CyaA to the CD11b/CD18 receptor distributed in the bulk phase of cell membrane, a ‘translocation intermediate’ of CyaA would insert into the cytoplasmic membrane. It is assumed that in this ‘translocation intermediate’ a part of the AC domain is already inserted into the membrane and is shielded from the lipids by association with the amphipathic α-helical transmembrane segments of the hydrophobic domain of CyaA (residues 502–522, 529–549, 571–591, 607–627 and 678–698 [19,20]). This ‘translocation intermediate’ then forms a path conducting external Ca^{2+} ions across cellular membrane into the submembrane compartment of cells. Incoming calcium ions activate the Ca^{2+}-dependent protease calpain, located in the submembrane compartment, which produces cleavage of the talin tether. This liberates the toxin-receptor complex from association with actin cytoskeleton and mobilizes it for recruitment into lipid rafts. Within the specific lipid-ordered environment of cholesterol-rich lipid rafts, translocation of the positively charged AC domain across the cellular membrane is completed, driven by the negative gradient of membrane potential.

Deciphering this fine-tuned mechanism of toxin action on cell membrane fosters our understanding of the key role played by CyaA in virulence of Bordetella during the early phases of bacterial colonization of host respiratory mucosa. It allows to propose the following scenario. The produced CyaA targets the CD11b/CD18 receptor of incoming myeloid phagocytic cells, such as neutrophils, macrophages and dendritic cells [42]. As CyaA action does not depend on receptor-mediated endocytosis, the toxin recruited into lipid rafts can rapidly translocate its highly active AC enzyme domain across the cytoplasmic membrane of cells, in a process exhibiting a half-time of only about ~30 seconds [38]. Mobilization of toxin-receptor complexes into lipid rafts than promotes their clustering and potentially induces recruitment of cellular cAMP-responding elements, such as the protein kinase A anchored to AKAPs, the specific A-kinase anchoring scaffolds [43–45]. This would allow maximization of toxin action through subversive cAMP production in close vicinity of components of the cAMP-regulated PKA signaling pathway. This capacity to hijack the spatio-temporal regulation of cellular cAMP/PKA signaling would then endow CyaA with the high potency in paralyzing the central bacterial mechanisms employed by myeloid phagocytic cells. Indeed, few picomoles of CyaA (1 ng/ml or less) were previously reported to instantaneously suppress the oxidative burst capacity of neutrophils [46], or the phagocytosis of complement-opsinized particles by macrophages [2].

Several other bacterial protein toxins appear, indeed, to utilize lipid rafts as a portal of cell entry, exploiting as specific receptors directly certain raft components, such as cholesterol, sphingolipids or GPI-anchored proteins [47–50]. In contrast, we found here that CyaA associates with rafts only upon binding and mobilization (hijacking) of its receptor CD11b/CD18. Unless activated in the process of leukocyte activation, this β2 integrin is distributed diffusely over the entire cellular membrane. As outlined above, we show here that upon binding of CyaA the integrin relocates into lipid rafts, due to toxin-induced and calcium-activated cleavage of talin by calpain. Moreover, the recently discovered capacity of CyaA to bind N-linked oligosaccharides of CD11b/CD18 [24] might also play a role in this process. It is, indeed, plausible to propose that CyaA interaction with terminal sialic acid residues of glycan chains of raft sphingolipids might also be contributing to accumulation of the CyaA-CD11b/CD18 complex in lipid rafts, as well as it may contribute to clustering of lipid rafts containing CyaA later-on. An evidence for CyaA interactions with gangliosides can, indeed, be deduced from the previously observed inhibition of CyaA activity on macrophages by the presence of micromolar concentrations of free gangliosides, such as Gt1b [23].

It remains to be addressed in future studies if CyaA can form oligomeric pores also once engaged in interaction with the target cell membrane through binding of the CD11b/CD18 receptor and whether CyaA can form pore-forming oligomers also in phagocyte membrane. We have recently succeeded in demonstrating the presence of the long-predicted CyaA oligomers within the membrane of cells lacking the receptor CD11b/CD18, such as erythrocytes [10]. Vojtova-Vodolavova with co-authors (2009), indeed, showed that CyaA oligomers underlies the pore-forming activity of CyaA towards erythrocytes. However, despite significantly higher amounts of CyaA binding per single phagocyte cell through the CD11b/CD18 receptor, fairly high concentrations (>1 μg/ml) of the recombinant enzymatically inactive but fully pore-forming CyaA-AC− variants are needed to provoke lysis of cells like J774A.1 monocytes in several hours [8]. While this resistance to colloid-osmotic lysis is likely to be to large extent due to membrane recycling mechanisms and pore removal form phagocyte cytoplasmic membrane, it remains to be shown that CyaA can form oligomeric pores in leukocyte membrane as well.

The results presented here do not indicate any role of CyaA oligomers in promoting calcium influx, toxin mobilization into rafts, or AC enzyme translocation into CD11b^{+} phagocytes. Early dose-dependence studies indicated that the AC domain was delivered across target cell membrane by CyaA monomers. Indeed, toxin molecules with the AC domain cleaved-off by cytosolic proteases, upon AC translocation into cells, were detected exclusively in form of CyaA monomers within erythrocyte membranes and were excluded from the detected CyaA oligomers [10]. Moreover, we used here the CyaA-E570Q-K860R-AC− protein, which essentially lacks any pore-forming activity and fails to permeabilize the membrane of J774A.1 cells, thus being unlikely to form any CyaA oligomers.
**Figure 6.** Cholesterol depletion inhibits translocation of AC domain across target cytoplasmic membrane. (A) J774A.1 cells were kept in buffer or treated with 10 mM MJCD at 37°C for 30 min, before CyaA was added for additional 10 min. CyaA binding and AC domain translocation were determined as described in the legend to Fig. 3. Values represent the mean ± S.D. from four independent experiments performed in triplicates. An asterisk indicates a statistically significant difference (*, p<0.01; Student's t test). (B) J774A.1 cells were kept in buffer or pretreated with 10 mM MJCD at 37°C for 30 min and incubated with 1 nM CyaA for 10 min. Cell lysates were analyzed as in Fig. 1A. (C) J774A.1 cells were loaded with the Ca^{2+} probe Fura-2/AM prior to cholesterol extraction with 10 mM MJCD at 30°C for 30 min. CyaA-mediated Ca^{2+} influx was recorded as above. Standard deviations were calculated for mean values at indicated time points and the shown curves are representative of at least three independent experiments. (D) J774A.1 cells grown on coverslips were kept in buffer or pretreated with 10 mM MJCD at 37°C for 30 min. Membrane distribution of
fluorescently labeled CyaA-AC' (6 nM, green) and CtxB (5 μg/ml, red) was visualized as described in the legend to Fig. 2. (E) U937 cells were grown for 48 hours in RPMI medium supplemented with 10% fetal calf serum (standard serum) or with 10% lipoprotein-deficient serum from fetal calf (delipidated serum). CyaA binding and AC domain translocation were determined as described in the legend to Fig. 3. Values represent the mean ± S.D. from four independent experiments performed in triplicates. An asterisk indicates a statistically significant difference (*, p<0.01; Student’s t test). (F) U937 cells grown in standard or delipidated serum were incubated with 1 nM CyaA at 37°C for 10 min, cell lyzates were separated on sucrose density gradients and analyzed as in Fig. 1. (G) U937 cells grown in standard or delipidated serum were loaded with the Ca2+ probe Fura-2/AM (3 μM) at 25°C for 30 min, exposed to CyaA (17 nM) and the time course of Ca2+ entry was recorded as above. Standard deviations were calculated for mean values at indicated time points and the shown curves are representative of at least three independent experiments. (H) U937 cells cultured with standard or delipidated serum were mounted on polylysin-coated coverslips and membrane distribution of fluorescently labeled CyaA-AC' (6 nM, green) and CtxB (5 μg/ml, red) was visualized as described above.

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(Tables 1 and [51]). On the other hand, this construct is fully capable to translocate the AC domain into cytosol of CD11b-expressing J774A.1 cells, to promote calcium influx and to associate with DRM, or to co-localize with CtxB in coalesced rafts, respectively (cf. Fig. 2). It appears, therefore, unlikely that oligomerization plays a role in DRM association of CyaA.

We also observed here that the levels of binding of CyaA to CD11b-expressing cells were not affected upon cholesterol depletion of cell membrane, while the translocation of the AC domain across the membrane depended strongly on the cholesterol content. This suggests that by modulating the physical properties lipid bilayers, cholesterol was specifically supporting the translocation of the AC domain across cell membrane. Indeed, cholesterol removal was previously found to impair the residual penetration capacity of CyaA on artificial membranes and erythrocytes [52,53]. This goes well with the impact of cholesterol concentration on membrane fluidity, lateral phase separation, formation of liquid-ordered structures and the propensity of lipids to adopt the inverted hexagonal phase [54,55]. The same membrane properties would also be expected to support AC domain translocation into cells by lowering the energy barrier for polypeptide penetration into and across the lipid bilayer [56]. It is plausible to speculate that membrane translocation of the AC domain requires the presence of cholesterol-dependent liquid-ordered (l0) phase, in which the acyl chains of lipids are tightly packed, while the individual lipid molecules have a high degree of lateral mobility. The relative mobility of lipids in l0 domains represents, indeed, a likely prerequisite for passage of the AC domain across lipid bilayer. A high condensation and immobility of lipids in liquid-disordered (ld)-phase domains would, in turn, be expected to interfere with AC polypeptide translocation. The requirement for sufficient membrane fluidity for AC translocation to occur is also indicated by the block of AC translocation at 4°C [38].

Recently, we demonstrated that AC domain translocation across target cell membrane is accompanied by entry of Ca2+ ions into cells. Moreover, the AC domain polypeptide as such was found to participate in formation of the transiently opened calcium influx path in cell membrane [18]. Here, we used the 3D1 MAb recognizing a distal segment of the AC domain and show that blocking of AC domain translocation across cell membrane can lock

Figure 7. AC domain of CyaA translocates across cellular membrane from lipid rafts. (A) J774A.1 cells were incubated at 37°C for 10 min with 1 nM individual CyaA proteins (upper panel) or with their 1:1 mixtures (lower panel). CyaA and biotinylated CyaA-E570K+E581P (CyaA-E570K+E581P-biotin) were detected in gradient fractions using 9D4 and streptavidine, respectively. (B) J774A.1 cells were incubated at 37°C for 10 min with the indicated pairs of proteins (12 nM) mixed in a 1:1 molar ratio. Binding of CyaA-E570K+E581P-biotin was determined as the amount of total cell-associated AC enzyme activity upon exposure of cells to the protein mixtures and expressed as relative value. Enhancement of the residual capacity of CyaA-E570K+E581P to translocate AC domain into J774A.1 cell cytosol (1.8±0.5% of intact CyaA activity) was measured by determination of intracellular cAMP amounts accumulated in 10⁶ cells upon exposure for 10 min at 37°C to the indicated 1:1 protein mixtures (12 nM). The values represent the mean ± S.D. from four independent experiments performed in duplicate. An asterisk indicates a statistically significant difference (*, p<0.01; Student’s t test).
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CyaA in a ‘translocation intermediate’ conformation forms a path for Ca\(^{2+}\) influx across cell membrane (cf. Fig. 3). Moreover, this ‘translocation intermediate’ was found to be recruited into lipid rafts (Fig. 3). The sum of the data hence allows us to answer the question what happens first, whether calcium influx precedes toxin mobilization into rafts, or whether recruitment of CyaA into rafts precedes calcium influx and AC translocation.

We showed here that calpeptin-mediated inhibition of calcium-activated processing of talin by calpain yields (i) inhibition of CyaA recruitment into rafts and (ii) it inhibits AC translocation across membrane. Collectively, hence, these results strongly suggest that the transient influx of Ca\(^{2+}\) into cells accompanies the earliest step of membrane insertion of the toxin ‘translocation intermediate’. This would preclude be essential for subsequent recruitment of CyaA into lipid rafts, whereupon AC translocation is accomplished.

It remains, however, to be determined what is the threshold of the calcium signal required for initiation of talin cleavage and mobilization of CyaA into lipid rafts. Two major isoforms of calpain have, indeed, been so far identified in eukaryotic cells. The calpain I (\(\mu\)-calpain) is activated at \(\mu\)M Ca\(^{2+}\) concentrations, while calpain II (m-calpain) only responds to mM concentrations of Ca\(^{2+}\) [57]. Here we observed that CyaA relocalization into DRM occurred at 1 nM toxin concentration, which is about two-times less than the lowest CyaA concentrations still allowing to elicit a [Ca\(^{2+}\)]\(_{i}\) increase detectable in cells by the Fura-2/AM probe [18]. Moreover, only influx of extracellular Ca\(^{2+}\) ions into cells, and not the elevation of cytosolic [Ca\(^{2+}\)]\(_{i}\), due to Ca\(^{2+}\) release from intracellular stores, enabled the accumulation of CyaA in DRM (cf. Fig. 4). This differs importantly from the mechanism reported for localization of the leukotoxin (LtxA) of *Actinobacillus actinomycetemcomitans* into rafts. LtxA binds yet another \(\beta\)-integrin of human leukocytes, the LFA-1 or CD11a/CD18 heterodimer. Moreover, LtxA appears to first adsorb on cell membrane of T lymphocytes in a receptor-independent manner, to trigger, somehow the store-operated elevation of cytosolic [Ca\(^{2+}\)]\(_{i}\), to induce talin cleavage, and upon relocation into rafts, the Ltx clusters with LFA-1 within rafts to promote cell lysis [58].

With CyaA, all the Ca\(^{2+}\) ions entering macrophage cytoplasm due to toxin action appear to come from extracellular medium [18]. It is generally accepted that there exists a gradient of about four orders of magnitude in Ca\(^{2+}\) concentrations between the external medium (\(\sim\)2 mM) and cell cytosol (\(\sim\)100 nM). Therefore, numerous Ca\(^{2+}\)-buffering proteins accumulate beneath the inner face of cell membrane, accounting for formation of local Ca\(^{2+}\) gradients and controlling signaling induced by alterations of Ca\(^{2+}\) concentrations in the submembrane compartment. These concentrations can, indeed, be still much higher, and rise more rapidly, than the bulk Ca\(^{2+}\) levels in cell cytosol [59]. Therefore, it is likely that even an importantly lower CyaA concentration than used here (1 nM = 176 ng/ml), may still be generating sufficiently high local Ca\(^{2+}\) signal beneath cell membrane in order to promote activation of \(\mu\)-calpain at the inner face of cell membrane. It appears, thus, plausible to assume that mobilization of CyaA into rafts in phagocyte membrane, and translocation of the AC domain from rafts directly into the cytosolic compartment of phagocytes, are indeed taking place also during natural *Bordetella* infections *in vivo*. This would account for the remarkable efficacy of CyaA in disarming the sentinel cells of the host innate defense.

**Materials and Methods**

**Expression and purification of CyaA-derived proteins**

Intact recombinant CyaA and its mutant variants were expressed and purified as previously described [19]. Except of pro-CyaA, the CyaA proteins were produced in *E. coli* XL1-Blue in the presence of the co-expressed toxin-activating acyltransferase...
CyaC, as previously described [20]. Lipopolysaccharide was eliminated by repeated 60% isopropanol washes of CyaA bound to the Phenyl Sepharose resin [60]. This reduced the final endotoxin content below 50 EU/mg of purified protein, as determined by the Limulus amebocyte lysate assay (QCL-1000, Cambrex, NJ, USA). For fluorescence microscopy, the CyaA proteins were labeled while bound to Phenyl-Sepharose resin during the final purification step. Briefly, the CyaA eluates from a DEAE-Sepharose column (GE Healthcare) in 50 mM Tris-HCl (pH 8), 0.2 mM CaCl₂, 200 mM NaCl, were eluted 1:4 with a buffer containing 50 mM Tris-HCl (pH 8), 1 M NaCl, and 1 mg of CyaA was loaded on an 0.5 ml Phenyl-Sepharose column. The columns were extensively washed with 0.1 M sodium bicarbonate (pH 9), 1 M NaCl, and the CyaA-Alexa Fluor 488 conjugate was eluted from the Phenyl Sepharose resin [60].

Depletion of cholesterol

J774.A1 cells were incubated in DMEM supplemented with 10 mM methyl-β-cyclodextrin (MβCD) at 37°C for 30 min. Cholesterol-depleted U937 cells were obtained upon growth in RPMI medium supplemented with 10% of delipidated serum (lipoprotein-deficient serum from fetal calf, Sigma) for 48 h. Cholesterol content was determined using an Amplex Red Cholesterol Assay Kit (Molecular Probes, Invitrogen) according to manufacturer’s instructions. Viability of cells was tested by trypan blue staining and no significant cell death occurred upon cholesterol extraction.

Fluorescence microscopy

U937 cells were grown in media with 10% standard, or delipidated serum, and were mounted on polylysine-coated coverslips prior to incubation with labeled proteins. J774.A1 cells (5.10⁶) were grown directly on coverslips (ϕ12 mm) and incubated with Alexa Fluor 488-labeled CyaA proteins (6 nM) at 37°C for 10 min, before cells were washed and 5 μg/ml of Alexa Fluor 594-labeled cholera toxin subunit B (CtxB) was added for additional 5 min. The unbound proteins were washed-off with ice-cold PBS, cells were fixed with 4% paraformaldehyde in PBS at 25°C for 20 min, and mounted in Moviol solution (Sigma). Fluorescence images were taken using a CellB Imaging Station (Olympus, Hamburg, Germany) based on Olympus IX 81 fluorescence microscope, using a 100 x oil immersion objective (N.A. 1.3). Digital images were processed using ImageJ software.

Antibodies, reagents, cell lines, SDS-PAGE, BN-PAGE and Western blotting

See Protocol S1 for full description.

Isolation of detergent-resistant membranes

Detergent-resistant membranes (DRM) were separated by flotation in discontinuous sucrose density gradients. Briefly, J774.A1 cells (2.10⁶) were washed with prewarmed DMEM and incubated with 1 nM CyaA proteins at 37°C for 10 min. Cells were washed with ice-cold phosphate-buffered saline (PBS), scraped from the Petri dish and extracted at 4°C for 60 min using 200 μl of TBS buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 1% Triton X-100, 1 mM EDTA, 10 mM NaF and a Complete Mini protease inhibitor cocktail (Roche, Basel, Switzerland). The lyzates were clarified by centrifugation at 250 x g for 5 min and the post-nuclear supernatants were mixed with equal volumes of 90% sucrose in TBS. The suspensions were placed at the bottom of centrifuge tubes and overlayed with 2.5 ml of 30% sucrose and 1.5 ml of 5% sucrose in TBS. Membrane flotation according buoyant density was achieved by centrifugation at 150,000 x g in a Beckman SW60Ti rotor for 16 h at 4°C. Fractions of 0.5 ml were removed from the top of the gradient.

Ca²⁺ influx into cells

Calcium influx into J774.A1 and U937 cells was measured as previously described [18]. Briefly, cells were loaded with 3 μM Fura-2/AM (Molecular Probes) at 25°C for 30 min and the time course of calcium entry into cells induced by addition 3 μg/ml of CyaA proteins was determined as ratio of fluorescence intensities (excitation at 340/380 nm, emission 505 nm), using a FluoroMax-3 spectrofluorometer equipped with DataMax software (Jobin Yvon Horriba, France).

Supporting Information

Figure S1  Cholesterol depletion does not affect the tight binding of CyaA to CD11b/CD18. J774.A1 (10⁶) cells were pretreated with 10 mM MβCD at 37°C for 30 min and placed on ice before CyaA or pro-CyaA were added at indicated concentrations. Proteins were allowed to bind CD11b/CD18 for 30 min at 4°C, before 30 nM biotinylated CyaA was added for another 30 min on ice. Cells were washed, stained with phycoerythrin-streptavidin conjugate and amounts of bound biotinylated CyaA were determined by flow cytometry. Results are expressed as relative binding of biotinylated CyaA according to the formula = (sample binding)/(maximum binding)x100. Data shown are the mean ± S.D. from three independent experiments performed in duplicates.
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Author Contributions

Conceived and designed the experiments: LB JM RF PS. Performed the experiments: LB JM RF. Analyzed the data: LB JM. Wrote the paper: LB PS.

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