FEBS Letters 581 (2007) 1287–1296

Differential requirement for the translocation of clostridial binary toxins: Iota toxin requires a membrane potential gradient

Maryse Gibert[a], Jean Christophe Marvaud[a], Yannick Pereira[a], Martha L. Hale[b], Bradley G. Stiles[b], Patrice Boquet[c], Christophe Lamaze[d], Michel R. Popoff[a,*]

[a] *Bactéries Anaérobies et Toxines, Institut Pasteur, Paris, France*
[b] *Toxinology Division, Department of Immunology and Molecular Biology, US Army Medical Research Institute of Infectious Diseases, Frederick, MD 21702-5011, USA*
[c] *INSERM U452, Faculté de Médecine, Nice, France*
[d] *Laboratoire Trafic et Signalisation, UMR144 Curie/CNRS, Institut Curie, 26 Rue d'Ulm, Paris, France*

Received 15 September 2006; revised 14 December 2006; accepted 13 February 2007

Available online 28 February 2007

Edited by Pascale Cossart

Abstract **Clostridial binary toxins, such as *Clostridium perfringens* Iota and *Clostridium botulinum* C2, are composed of a binding protein (Ib and C2-II, respectively) that recognizes distinct membrane receptors and mediates internalization of a catalytic protein (Ia and C2-I, respectively) with ADP-ribosyltransferase activity that depolymerizes the actin cytoskeleton. After internalization, it was found that C2 and Iota toxins were not routed to the Golgi apparatus and exhibited differential sensitivity to inhibitors of endosome acidification. While the C2-I component of C2 toxin was translocated into the cytosol from early endosomes, translocation of the Ia component of Iota toxin occurred between early and late endosomes, was dependent on more acidic conditions, and uniquely required a membrane potential gradient.**

Keywords: Iota toxin; C2 toxin; Membrane potential; Translocation; Endocytosis; *Clostridium*

1. Introduction

Clostridial binary toxins are actin-modifying, ADP-ribosylating proteins produced by several *Clostridium* species and divided into two families. The Iota-family encompasses Iota toxin from *Clostridium perfringens* type E, *Clostridium spiroforme* toxin and *Clostridium difficile* ADP-ribosyltransferase. C2 toxin is produced by *Clostridium botulinum* types C as well as D, and is the sole representative of the C2-family. The clostridial binary toxins share a common structure, consisting of two independent proteins corresponding to the enzymatic and binding components. The binding component (Ib for Iota toxin and C2-II for C2 toxin) is synthesized as a nontoxic precursor (100 and 80 kDa, respectively) that is proteolytically activated into the mature form (80 and 60 kDa, respectively) after removal of a 20 kDa N-terminal peptide. These binding components recognize distinct, yet unidentified, cell-surface receptors and mediate cytosolic internalization of the corresponding enzymatic component. In contrast to the enzymatic component of C2 toxin (C2-I), that of Iota toxin (Ia) requires proteolytic activation via cleavage of 9 to 13 N-terminal amino acids [1]. Ia catalyzes the mono ADP-ribosylation of globular actin from muscle and non-muscle types at residue R177, whereas C2-I activity is restricted to non-muscular actin [2,3]. These toxins are responsible for a complete disorganization of actin filaments and cell rounding. Toxin components of the Iota-family are immunologically related and can be interchanged to form biologically-active, hybrid toxins. Thereby, Ib can internalize the enzymatic components from either *C. spiroforme* or *C. difficile* toxin. In contrast, C2 toxin which is structurally and functionally related to the Iota-family of toxins, does not immunologically cross react or associate with Iota-family components to reconstitute a biologically functional toxin [4,5].

Many toxins are active only at the intracellular level, and cell entry is therefore the first critical step for intoxication. Once inside a cell, bacterial toxins can exploit various mechanisms to reach the cytosol and their substrate. Monomeric toxins such as diphtheria toxin (DT) interact with endosomal membranes through a hydrophobic domain, and translocation of the unfolded enzymatic domain into the cytosol requires a pH gradient. The enzymatic components (edema and lethal factors) of the respective binary edema and lethal toxins from *Bacillus anthracis* are also translocated into the cytosol at low pH. This is accomplished through a protein channel formed after heptamerization of binding components (protective antigen, PA) in lipid rafts and subsequent internalization into endosomes via a clathrin-dependent route [6,7].

In contrast, other toxins such as cholera toxin (CT), Shiga toxin, and ricin are not translocated from endosomes into the cytosol. Instead, these toxins are routed through retrograde transport via the Golgi apparatus into the endoplasmic reticulum (ER), and finally the enzymatic domain is translocated into the cytosol without acidification. The exotoxin A from *Pseudomonas aeruginosa* utilizes yet another route, as the toxin is modified in an acidic compartment and subsequently translocated from another non-acidic organelle [8]. Although exotoxin A is transported to the Golgi apparatus and ER, its effects are impaired upon blocking the acidification of intracellular compartments [9–12].

To date, little is known about the entry and translocation process of clostridial binary toxins. Iota and C2 toxins enter

*Corresponding author. Fax: +33 1 40 61 31 23.
E-mail address: mpopoff@pasteur.fr (M.R. Popoff).

0014-5793/$32.00
doi:10.1016/j.febslet.2007.02.041

Report Documentation Page

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
3 APR 2007	**N/A**	**-**

4. TITLE AND SUBTITLE
Differential requirement for the translocation of clostridial binary toxins: iota toxin requires a membrane potential gradient. FEBS Letter 581:1287 - 1296

5a. CONTRACT NUMBER
5b. GRANT NUMBER
5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Gibert, M Marvaud, J-C Pereira, Y Hale, ML Stiles, BG Boquet, P Lamaze, C Popoff, MR

5d. PROJECT NUMBER
5e. TASK NUMBER
5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)

10. SPONSOR/MONITOR'S ACRONYM(S)
11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release, distribution unlimited

13. SUPPLEMENTARY NOTES
The original document contains color images.

14. ABSTRACT
Clostridial binary toxins, such as Clostridium perfringens Iota and Clostridium botulinum C2, are composed of a binding protein (Ib and C2-II, respectively) that recognizes distinct membrane receptors and mediates internalization of a catalytic protein (Ia and C2-I, respectively) with ADP-ribosyltransferase activity that depolymerizes the actin cytoskeleton. After internalization, it was found that C2 and Iota toxins were not routed to the Golgi apparatus and exhibited differential sensitivity to inhibitors of endosome acidification. While the C2-I component of C2 toxin was translocated into the cytosol from early endosomes, translocation of the Ia component of Iota toxin occurred between early and late endosomes, was dependent on more acidic conditions, and uniquely required a membrane potential gradient.

15. SUBJECT TERMS
Clostridium botulinum, botulism, iota toxin, membrane-activated compounds, translocation to cytosol, membrane potential gradient

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			
unclassified	**unclassified**	**unclassified**	**SAR**	**10**	

cells by receptor-mediated endocytosis and require an acidification step before translocation into the cytosol [13–15]. Since the effects of Iota toxin are not altered by chloroquine or monensin, two alkalinizing agents that interfere with intracellular trafficking of C2 toxin, it is likely that Iota toxin uses a different mechanism for entry and further intracellular transport [16].

Our results reveal that internalization of Iota and C2 toxins by a cell brings these proteins into an acidic endosomal compartment. In contrast to C2 toxin, a membrane potential is necessary for translocating Iota toxin into the cytosol and to our knowledge, this represents a genuinely novel process never described for cellular intoxication by any bacterial toxin.

2. Materials and methods

2.1. Materials

Amiloride, chloroquine (Chl), monensin (M), bafilomycin A1 (Baf), brefeldin A (BFA), chlorpromazin (Chp), filipinIII, nigericin (Ni), concanamycin (Con), ammonium chloride, and nocodazole were from Sigma.

2.2. Bacterial strains and toxin preparations

C. perfringens strains were grown in broth containing 30 g of Trypticase, 20 g of yeast extract, 5 g of glucose, and 0.5 g of cysteine-HCl/l (pH 7.2) under anaerobic conditions. Ib and Ia components were produced from *C. perfringens* strain 667 (lecithinase-, enterotoxin-, beta-, epsilon-, and Iota-negative strain) harboring the recombinant plasmid pMRP384 or pMRP147, respectively [1,17]. In the same way, C2-II and C2-I were produced from *C. perfringens* strain 667 harboring the recombinant plasmid pMRP885 or pMRP845, respectively. Iota and C2 toxin components were purified as described previously, and respectively α-chymotrypsin- or trypsin-activated Ib or C2-II, were used in this study [1]. Fluorescent Ib and C2-II were labeled with Cy3 (Amersham Biosciences) or Alexa555 (Molecular Probes) according to the manufacturer's recommendations. There was no significant loss of biological activity with fluorescent Ib and C2-II as discerned by cytotoxicity assays with the corresponding enzymatic components.

2.3. Cell cultures and assays with inhibitors

Vero cells were confluently grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS) in 96-well plates for Iota and C2 toxin activity assays. Cells were pretreated for 30 min with the different drugs at the indicated concentrations in DMEM-10% FCS. For nocodazole experiments, cells were incubated in DMEM-10% FCS containing 30 μM nocodazole on ice for 30 min. The depolymerization of microtubules induced by nocodazole was monitored by immunofluorescence with an anti-tubulin monoclonal antibody (Amersham). Serial twofold dilutions of Iota and C2 toxins containing equimolar concentrations of enzymatic and binding components, starting at 10^{-7} M, were then added to cells and incubated at 37 °C for the indicated times. The cytopathic effects of Iota or C2 were assessed by actin depolymerization as described below.

2.4. Measurement of filamentous actin

The amount of filamentous actin was measured using a modification of a previously described method [18,19]. After toxin treatment, cells grown in 96-well plates were then washed with phosphate buffered saline (PBS) and fixed with paraformaldehyde (3%) in PBS for 20 min at room temperature. After two washes in PBS, the cells were stained with 175 ng/ml rhodamine-labeled phalloidin (Molecular Probes) in PBS containing 0.5% bovine serum albumin (BSA) and 0.5 mg/ml saponin for 1 h at room temperature. After washing, rhodamine phalloidin was extracted with methanol (100 μl/well) overnight at −20 °C, and fluorescence was measured with a spectrometer (Fluoroskan, Labsystems) at 544 nm ($\lambda_{excitation}$) and 590 nm ($\lambda_{emission}$). Results are expressed as the percentage of filamentous actin content in cells treated with toxins, versus untreated cells. An additional control for actin polymerization included Latrunculin (Sigma), a sponge-derived toxin that binds monomeric actin and prevents formation of actin filaments.

2.5. Acidic pulse experiments

Vero cell monolayers were incubated on ice for 30 min in DMEM-25 mM HEPES-10% FCS containing 100 nM Baf and serial twofold dilutions of 10^{-7} M Iota or C2 toxin. The cells were then washed three times with cold PBS and incubated with 100 nM Baf for 10 min at 37 °C in PBS at pH 4, 5, 6 or 7.2, as described previously [20]. Cells were further incubated for 2 h in DMEM-10% FCS containing Baf, and the actin filament content was determined as described above. Controls corresponded to cells incubated with toxin in PBS, pH 7.2 that did not contain Baf.

2.6. Diphtheria toxin (DT) and ricin toxicity assays

DT and ricin toxicity were assayed according to Lemichez et al. [21]. Briefly, Vero cells were grown to confluence in 24-well plates with DMEM-10% FCS. Medium was then discarded and the cells incubated with pre-warmed, leucine-free DMEM (Gibco). After 30 min at 37 °C, the different drugs were added and further incubated with cells for 30 min at 37 °C. DT or ricin (400 ng/ml) was added to the cells for 2 h at 37 °C. The medium was replaced by leucine-free DMEM containing 1 μCi [^{14}C] leucine (Dupont NEN). After 1 h at 37 °C, the cells were washed three times with 1 ml of 10% trichloro-acetic acid. The cells were solubilized in 200 μl of 0.2 M NaOH, and 50 μl were then mixed with 4 ml of scintillation fluid before recording the radioactivity. Results are expressed as a percentage of inhibited protein synthesis relative to control preparations not incubated with DT or ricin.

2.7. Immunofluorescence

Non-confluent Vero cells grown on cover slides were incubated on ice for 30 min in DMEM-25 mM HEPES-10% FCS containing 10^{-7} M Ib-Cy3 or C2-II-Cy3. Following three washes with cold DMEM, cells were incubated in prewarmed (37 °C) DMEM-25 mM HEPES-10% FCS with or without inhibitors for the indicated times. Cells were washed with PBS, fixed with 4% paraformaldehyde in PBS for 20 min, washed with PBS, and free radicals were quenched by incubation with 50 mM NH_4Cl in PBS. Cells were then permeabilized for 5 min with 0.2% Triton X-100 in PBS and incubated with Texas Red-phalloidin. After washing in PBS, coverslips were mounted in Mowiol (Calbiochem), and observed by confocal fluorescence microscopy.

2.8. SDS–PAGE and Western blot analysis

Vero cells incubated with 10^{-7} M Ib with or without inhibitors as indicated, were washed with cold DMEM-FCS and lysed in 20 mM Tris–HCl, pH 7.5 containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.2 mg/ml BSA, and protease inhibitors (PMSF, leupeptin and pepstatin). Cell lysates were centrifuged (5 min at 800 × *g*) to remove nuclei and the supernatant was briefly sonicated on ice with a microtip probe (10 s at 20%). Samples (100 μg total protein each) were mixed with SDS Laemmli buffer without reducing agent or heating, and electrophoresed on a 4–15% acrylamide gradient gel (Ready gel, BioRad). Separated proteins were transferred to nitrocellulose, incubated with PBS containing 5% skim milk, and probed with anti-Ib antibodies as previously described [22,23]. Estimation of percent Ib oligomers versus Ib monomers, was done by densitometry scanning using Image J (NIH).

3. Results

3.1. Endocytosis of fluorescent Iota and C2 toxin binding components into Vero cells

We have shown that Ib can be transcytosed through CaCo-2 cells independently of Ia [24,25]. Thus, we monitored the entry of Ib or C2-II into Vero cells using fluorescently labeled toxin components. Incubation of Vero cells with Ib-Cy3 or

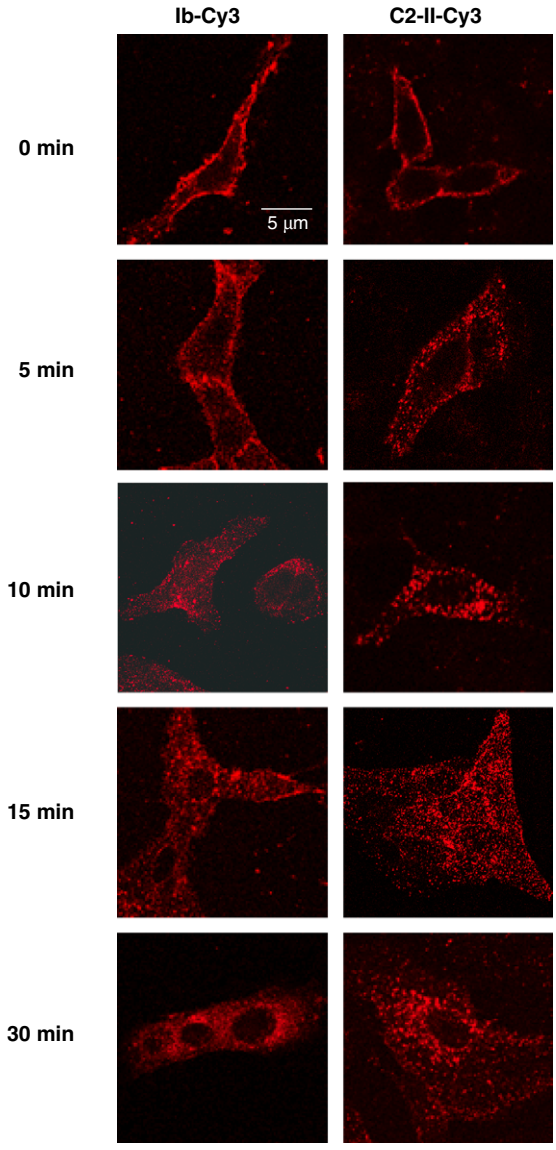


Fig. 1. Endocytosis of fluorescent Ib and C2-II into Vero cells. Vero cells were incubated on ice with 10^{-7} M Ib-Cy3 or C2-II-Cy3 for 30 min. After three washes with cold DMEM, cells were transferred to DMEM prewarmed at 37 °C. Typical staining of the cell periphery was visualized after incubation of cells with Ib-Cy3 or C2-II-Cy3 at 4 °C. Transfer of cells to prewarmed medium induced rapid endocytosis of fluorescent Ib and C2-II into vesicular compartments first located at the cell periphery, and then in the perinuclear area over time.

C2-II-Cy3 at 4 °C resulted in plasma membrane staining in agreement with the binding of toxin components to a cell-surface receptor [26,27] (Fig. 1). Upon transfer of cells from 4 °C to 37 °C, Ib and C2-II molecules were rapidly internalized. After 5 min at 37 °C, most of the fluorescent Ib and C2-II were observed in intracellular vesicles namely localized near the plasma membrane. After 10 min, endocytosed Ib and C2-II were no longer associated with the cell membrane and observed in vesicular structures scattered throughout the cytosol. Over time, intracellular compartments stained with Iota and C2 toxin binding components were accumulated in the perinuclear area, whereas staining of peripheral compartments clearly disappeared (Fig. 1).

3.2. Cytosolic entry of Iota and C2 toxins requires an acidic pulse

Historically, the study of toxin transport has allowed the identification of several novel intracellular pathways [8]. For instance, DT is delivered into the cytosol from early endosomes following acidification of endocytic vesicles [9]. In contrast, Shiga toxin and ricin use a more complex retrograde route to reach the Golgi apparatus and ER, but these toxins do not require acidification for translocation into the cytosol [28,29]. Iota and C2 toxins do require an acidic pH for cytosolic entry from endosomes, but the pH requirements for each are evidently different [13–17,30]. Here, we studied the cytotoxic effects of Iota and C2 toxins following their cellular uptake by monitoring the depolymerization of actin filaments induced by intracellular enzymatic components. We used a quantitative assay that measures the amount of filamentous actin in order to more precisely examine the potential differences existing between cytosolic entry of C2 and Iota toxins, that includes: (1) pH requirements for translocation of intracellular biologically active Ia and C2-I; and (2) differential protective effects on cells of chloroquine and monensin that were reported previously [16,30]. A Latrunculin control for actin polymerization was used to further confirm the C2 and Iota toxin result from these experiments. There was a similar dose–response curve evident for these toxins on Vero cells (data not shown).

The alkalinizing agents chloroquine and monensin are, respectively a proton trap and an ionophore for monovalent cations that reverse the proton gradient through the endosomal membrane [31]. These drugs, when used at concentrations that block the activity of DT (Fig. 2C), efficiently prevented actin depolymerization induced by C2 (Fig. 2B), but not Iota, toxin (Fig. 2A). Even a 20-fold higher concentration of monensin (100 μM) did no prevent Iota toxin-induced cytotoxicity (data not shown). Another ionophore, nigericin, also inhibited the cytoskeletal perturbations of C2 toxin in Vero cells, but again there was no effect on Iota toxin (Fig. 2A and B).

In contrast to the ionophore drugs, bafilomycin A1 (Baf) or concanamycin prevent endosomal acidification by selective inhibition of the vacuolar-ATPase [32], and both were as efficient at blocking the effects of Iota or C2 toxin on actin (Fig. 2A and B). It is likely that the differential sensitivity of Iota and C2 toxins towards these drugs reflects differences in translocation and/or intracellular pathways used by these related toxins.

Since Iota toxin activity was more resistant to the inhibitors of endosomal acidification than C2 toxin, quantitative measurement of filamentous actin was performed with acidic pulses in Vero cells pretreated with Baf and subsequently incubated with Iota or C2 toxin. Baf inhibits the normal entry pathway of toxin from acidified endosomes. However, a brief exposure of cells to a low pH mimics intravesicular acidification and thus permits translocation of cell-bound toxin directly into the cytosol through the plasma membrane [9].

Figs. 3A and B show that the normally protective effects of Baf on actin depolymerization induced by Iota or C2 toxin were overridden when the cells were bathed for 10 min in a buffer at pH 6 or less, in agreement with previous work [14,15,33]. We found that a pH of 4 was more efficient than a higher pH for reversing the inhibitory effect of Baf on C2 and Iota toxin (Fig. 3A and B), which is similar to the pH value (below pH 5.3) found for the direct entry of DT from

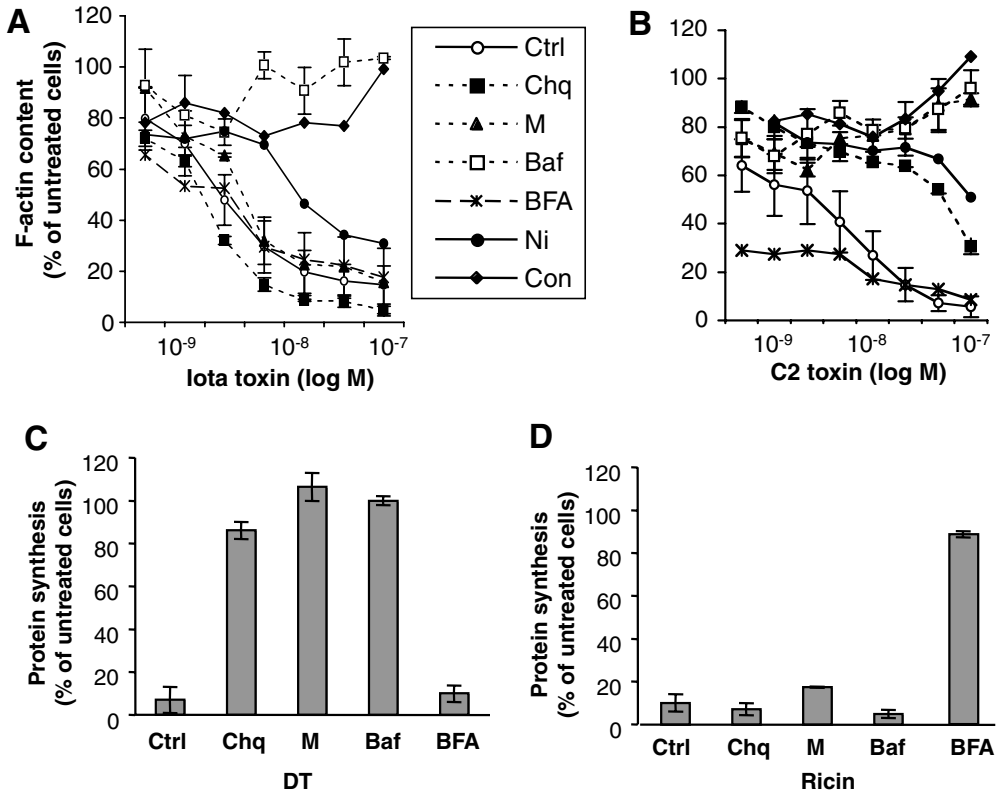


Fig. 2. Effects of pharmacological drugs on Iota and C2 toxin-induced actin depolymerization in Vero cells. Actin filament content was measured in Vero cells pretreated either with chloroquine (0.1 mM; Chq), monensin (5 μM; M), bafilomycin A1 (100 nM; Baf), brefeldin A (2.5 μg/ml; BFA), nigericin (10 μM; Ni), concanamycin (20 nM; Con), or media alone (Ctrl) and exposed to serial two-fold dilutions of Iota toxin (A) or C2 toxin (B). Filamentous actin content was measured as described in Section 2. Controls for the activity of chloroquine (0.1 mM), monensin (5 μM), bafilomycin A1 (700 nM), and brefeldin A (2.5 μg/ml) were DT (5 nM) (C) and ricin (5 nM) (D) as measured by inhibition of protein synthesis in Vero cells. Data are means ± S.D. ($n = 5$).

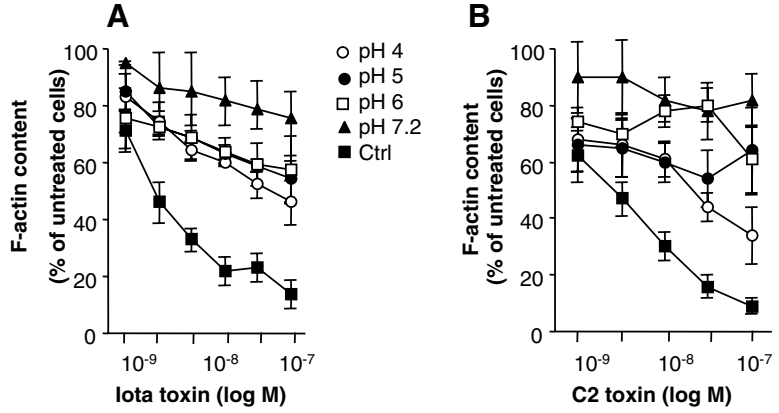


Fig. 3. Requirement of an acidic pulse for Iota and C2 toxin entry into Vero cells. For acidic pulse experiments, Vero cell monolayers were washed with cold DMEM-25 mM HEPES-10% FCS and incubated on ice for 30 min in the same medium containing 100 nM Baf plus serial twofold dilutions of either Iota (A) or C2 (B) toxin. The cells were then washed three times to remove unbound toxin with cold PBS, and incubated in PBS containing Baf at a different pH for 10 min at 37 °C. Cells were briefly washed with cold PBS and incubated for an additional 2 h at 37 °C in DMEM-1% FCS containing Baf to prevent toxin entry by its normal route. Controls of toxin activity (■) were performed in cells treated with toxin, but not Baf, at pH 7.2. Data are means ± S.D. ($n = 5$).

the cell surface into the cytosol [20]. Our findings did not preclude that both toxins require the same low pH for optimal translocation of the enzymatic component, and in fact Blöcker et al. [15] discovered that Iota toxin requires a lower pH than C2 for entry directly from the cell surface into the cytosol, thus circumventing the normal endosomal pathway.

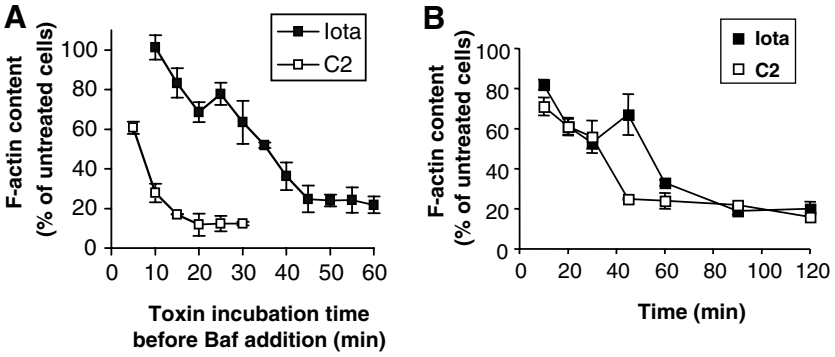


Fig. 4. Iota and C2 toxins use kinetically different trafficking pathways. (A) Vero cells were treated either with 10^{-7} M Iota (■) or C2 (□) toxin (time point "0"), and Baf (100 nM) was added at different time intervals after toxin incubation. The cells were further incubated at 37 °C for 2 h after the last addition of Baf, and F-actin content was assayed. Baf prevented the loss of actin filaments during a longer time period in cells incubated with Iota toxin versus C2 toxin. As a control (B), actin filament content was measured in Vero cells following Iota or C2 toxin entry triggered from the plasma membrane by an acidic pulse, thus bypassing the normal endocytic process blocked by Baf. Cells were pretreated with Baf (100 nM) for 30 min at 37 °C and then incubated with Iota or C2 toxin (10^{-7} M) in the presence of Baf for an additional 30 min at 37 °C. After washing, the cells were incubated with PBS (pH 4) for 10 min, and then in DMEM containing Baf. At the indicated times, actin filament content was measured spectrophotometrically via rhodamine-labeled phalloidin. These latter results revealed that Iota and C2 toxins possess quite similar kinetics and effects upon Vero cells when they enter the cytosol directly from the cell surface. Data are means ± S.D. ($n = 5$).

3.3. C2 and Iota toxins use kinetically different trafficking pathways

To further explore the link between acid-dependent entry into the cytosol and subsequent biological activity of these toxins, the following experiment was performed. The depolymerization of actin filaments was measured in Vero cells incubated with Iota or C2 toxin, followed by the addition of Baf at subsequent time intervals (Fig. 4A). When Baf was added 15 min or later after C2 toxin, there was no protection against actin depolymerization induced by the toxin. However, the time period required for the same protective effect by Baf was longer (~35–40 min) for Iota toxin. These results indicated that surface binding, endocytosis, intracellular trafficking and biological activity upon cells (i.e. actin depolymerization) of C2 toxin occurred overall more rapidly than that for Iota toxin.

Additionally, we have verified that the time required for translocation and then intracellular activity of Ia and C2-I are virtually identical (Fig. 4B). For that, Vero cells pretreated with Baf (100 nM) were incubated with Iota or C2 toxin, which thereby could not enter cells by their normal endocytic pathway. Toxin entry directly from the plasma membrane into the cytosol was triggered by an acidic pulse (pH 4, 10 min), and the kinetics of actin filament depolymerization were monitored over time (Fig. 4B).

Overall, these collective results strongly suggested that the endocytic process is shorter for C2 toxin versus Iota toxin. It is therefore possible that C2 toxin either uses a more efficient mechanism of translocation at low pH than Iota toxin, or each toxin can follow a distinct endocytic pathway to reach different intracellular compartments with an appropriate pH for translocation.

In addition to investigating the pH effects upon cytosolic entry of Iota and C2 toxins, two other drugs were used for protection studies in the quantitative actin assay. Brefeldin A (BFA), a Golgi disrupter and inhibitor of CT, ricin, and Shiga toxin transport, as well as nocodazole, a drug that disrupts tubulin filaments and blocks early to late endosomal trafficking, were both tested in Vero cells. Neither BFA nor nocodazole prevented actin modification by Iota or C2 toxin (Fig. 2A–D). These results are similar to those previously reported by Blöcker et al. [15] when using a cytotoxicity test. However, in contrast to Iota toxin, C2 toxin and DT, BFA did block ricin effects on cells (Fig. 2D) [34].

3.4. Both a proton and vesicle transmembrane potential are required for translocating Iota toxin

It was intriguing that potent inhibitors of vesicle acidification, such as monensin, did not prevent the cytosolic entry of Iota toxin. Recently, it was found that the fibroblast growth factor-1 (FGF-1) is translocated from vesicles possessing vacuolar proton pumps by means of a vesicular membrane potential. In this case, the translocation of FGF-1 is blocked by Baf, which presumably prevents both the pH and transmembrane potential gradients [35], however, monensin or lysomotropic agents such as ammonium chloride and chloroquine that only prevent endosomal acidification are ineffective. Translocation of FGF-1 is also prevented by the combination of monensin and valinomycin, which are respectively a neutral monovalent cation exchanger and electrogenic K^+ ionophore that abolish the transmembrane potential. Therefore, intravesicular H^+ are exchanged for K^+ by monensin, while valinomycin dissipates K^+ into the cytosol and thus abolishes the membrane potential [35]. When we tested a combination of monensin and valinomycin with various concentrations of Iota toxin, an inhibitory effect was observed (Fig. 5A).

The inhibition of FGF-1 translocation by Baf is overcome by monensin, due to activated Na^+/K^+-ATPase that exchanges K^+ for Na^+ and restores the membrane potential required for FGF-1 transport across membranes [35]. When using a low concentration of Baf (10 nM) that partially inhibited the effects of Iota toxin, addition of monensin blocked the depolymerization of actin induced by Iota in a concentration-dependent manner (Fig. 5B). A possible interpretation of these results was that vesicles where translocation of Iota toxin had occurred, were either devoid of, or had non-functional forms of, Na^+/K^+-ATPase. Low amounts of Baf were sufficient to inhibit the membrane potential, which was not restored by adding monensin and thus reactivating Na^+/K^+-ATPase. Additionally, the pH gradient was totally abolished by Baf and monensin. We have checked whether inhibition of the

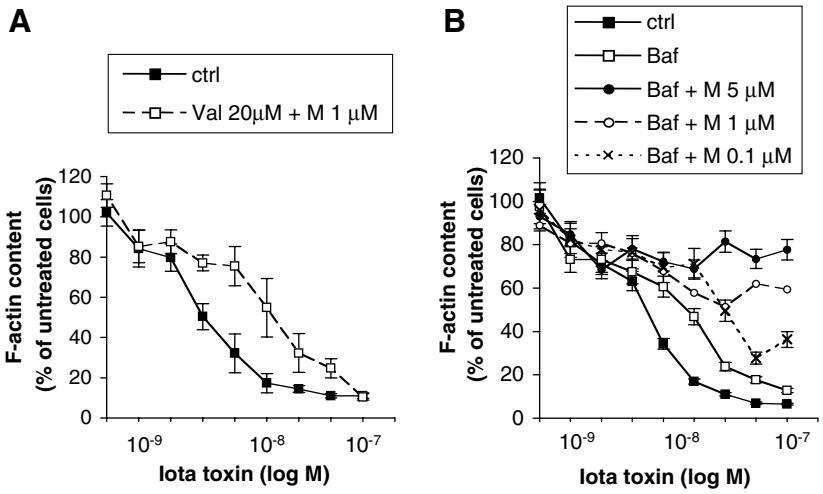


Fig. 5. Iota toxin requires a gradient of membrane potential to elicit its cytopathic effect. (A) Vero cells were pretreated with valinomycin (Val; 20 μM) and monensin (M; 1 μM), then incubated with Iota toxin. A combination of Val and M delayed the cytopathic effects induced by Iota toxin relative to cells without drugs (ctrl), or those pretreated with Val (20 μM) or M (1 μM) alone (not shown). (B) Cells were pretreated with Baf (10 nM) alone or in association with 5, 1, or 0.1 μM monensin (M) for 30 min at 37 °C, and then exposed to Iota toxin. Cells were incubated for an additional 2 h and the actin filament content was assayed. The combination of M with a sub inhibitory concentration of Baf prevented the Iota cytopathic effects in a dose-dependent manner. Data are means ± S.D. ($n = 3$).

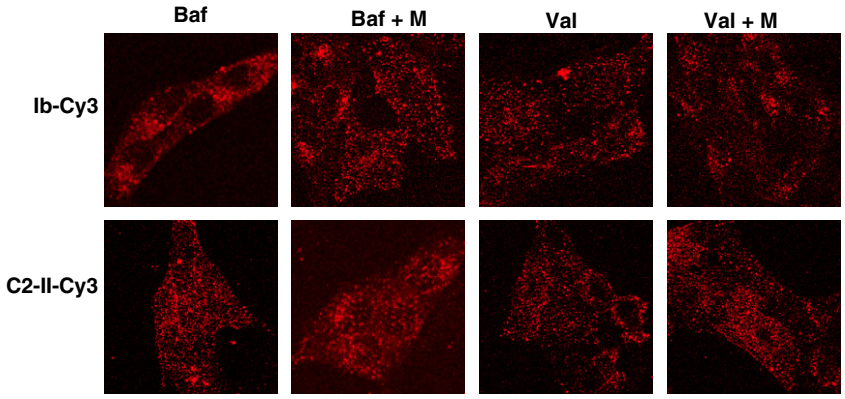


Fig. 6. Inhibition of endosome acidification and membrane potential does not impair endocytosis of Ib or C2-II. Vero cells were pretreated with 100 nM bafilomycin A1 (Baf), 100 nM Baf + 1 μM monensin (M), 20 μM valinomycin (Val), or 20 μM Val + 1 μM M. Binding of fluorescent Ib or C2-II was done at 4 °C, as previously described in Fig. 1, and cells were then transferred to prewarmed DMEM medium at 37 °C for 15 min. Endosome labeling induced by Ib-Cy3 or C2-II-Cy3 was not impaired by the drugs as compared to controls in Fig. 1.

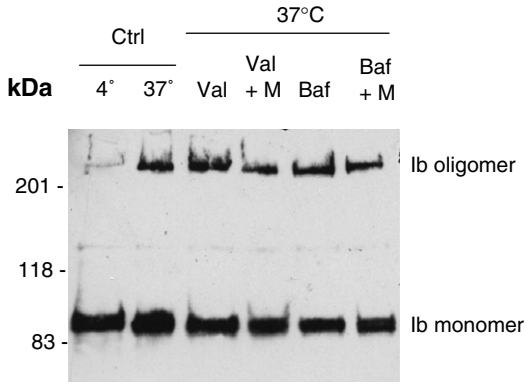
endosomal pH and/or transmembrane potential gradients impair endocytosis of the binding components. As shown in Fig. 6, either Baf and valinomycin, alone or in combination with monensin at concentrations that inhibit cytotoxicity, did not impair the uptake of fluorescent Ib or C2-II into intracellular vesicles after 15 min at 37 °C.

Overall, these experiments suggested that the translocation of Iota toxin required both a proton and membrane potential gradient, and this process occurred from endocytic carrier vesicles and late endosomes that possess a vacuolar-, but not a Na^+/K^+-ATPase [36].

3.5. *Formation of Ib functional channels is not impaired by inhibitors of endosome acidification and transmembrane potential*

A prerequisite step in Iota or C2 intoxication is the formation of functional Ib or C2-II pores through the endosomal membrane to enable passage of the corresponding enzymatic component into the cytosol [17,33]. For PA, which shares a significant amino acid sequence homology with Ib [37], functional pores occur at neutral or low pH and correlate with formation of SDS-resistant oligomers [38–40]. Further studies show that SDS-resistant PA heptamers are mainly formed in early endosomes [41]. However, formation of SDS-resistant Ib oligomers on Vero cells is not influenced by pH ranging from 4 to 9 [23]. Therefore, we investigated whether inhibitors of endosome acidification or transmembrane potential impair formation of SDS-resistant Ib oligomers in Vero cells.

As previously described [23], Ib incubated with Vero cells at 4 °C did not significantly form oligomers, whereas incubation at 37 °C induced a large proportion of SDS-resistant Ib oligomers dependent upon lipid rafts [22]. Baf, valinomycin, or combinations with monensin did not significantly impair formation of SDS-resistant Ib oligomers on Vero cells, at least at the drug concentrations used for these studies as shown by SDS-gel/Western blots and densitometry-based estimation of the oligomers rate (Fig. 7).



	4°C	37°C	Val	Val +M	Baf	Baf +M
Ib oligomer (%)	7.6 +/-3.1	30.3 +/-6.8	37.8 +/-12.1	29.3 +/-8.3	37.7 +/-9.5	35.6 +/-10.3

Fig. 7. Inhibition of endosome acidification and membrane potential does not impair Ib-dependent SDS-resistant oligomer formation. Vero cells were incubated in DMEM-FCS without (Ctrl) or with 20 μM valinomycin (Val), 20 μM valinomycin + 1 μM monensin (Val + M), 100 nM bafilomycin A1 (Baf), or 100 nM bafilomycin A1 + 1 μM monensin (Baf + M), for 30 min at 37 °C. Ib (10^{-7} M) was added to the culture medium with cells and incubated for an additional 10 min at 37 °C, except for control cells (no drug added) kept on ice prior, and after, the addition of Ib. Cells were lysed and 100 μg total protein per sample mixed with SDS-Laemmli buffer without reducing agent or heat. Electrophoresis was done on a 4–15% polyacrylamide gel, separated proteins were then transferred to nitrocellulose, and finally probed with anti-Ib antibodies. SDS-resistant oligomers were formed at 37 °C, but not 4 °C, and this large molecular weight complex was not impaired by inhibitors of endosome acidification and membrane potential. Quantitative percentages of Ib oligomer versus Ib monomer were determined with Image J (NIH) densitometry scanning from three independent experiments.

4. Discussion

Binding of Iota and C2 toxins to currently unidentified cell-surface receptors, is followed by their endocytosis into a vesicular/endosomal compartment from which they must escape to reach the cytosol and modify monomeric actin. Drugs that inhibit endosomal acidification protected cells from intoxication by C2 toxin as well as DT (Fig. 2B and C), and the anthrax toxins, as previously shown [13,14,16,30,42,43]. In contrast, Iota toxin differs from C2 and anthrax toxins since chloroquine, monensin, nigericin, or ammonium chloride did not inhibit its activity (Fig. 2A). *C. spiroforme* toxin, which is very highly related to Iota toxin, is also cytotoxic in the presence of chloroquine and ammonium chloride [44,45]. However, Iota and C2 toxins were equivalently blocked by two different vacuolar-ATPase inhibitors, Baf or concanamycin, and both toxins were internalized from the cell surface into the cytosol following an acidic pulse of Baf-treated cells (Fig. 3A and B), thus agreeing with previous data [14,15]. A pH gradient between the endosomes and cytosol is probably required for translocating Ia and C2-I, just as described for anthrax edema and lethal factors. A lower pH is seemingly required for translocating Iota, versus C2, toxin into the cytosol [15], and this perhaps could explain how weak bases, such as chloroquine, inefficiently protected Vero cells from Iota toxin activity. However, the inhibitory effects of chloroquine have been related not only to preventing endosomal acidification, but also binding of this drug to the vestibule area of the C2-II pore, which subsequently blocks the channel required for delivering enzymatic component into the cytosol [30,46,47]. Both chloroquine and tetraalkylammonium ions bind with high affinity to C2-II and PA, thus preventing toxicity, whereas these compounds bind with lower affinity to Ib and thus do not impair its biological activity [17,30,47,48]. Iota toxin effects were not prevented by monensin, which is an electroneutral exchanger of monovalent cations that only abolishes the pH gradient through endosomal membranes. Furthermore, inhibitors of endosomal acidification or membrane potential did not impair the assembly of SDS-resistant Ib oligomers in Vero cells which is a prerequisite step towards forming functional channels. It is noteworthy that Ib oligomers rapidly form in less than 1 min at 37 °C on Vero cells [23], and that Baf added subsequent to Iota toxin inhibited the latter's effect upon Vero cells (Fig. 4). This indicates that pH and/or membrane potential gradients between the endosomal compartment and cytosol are critically required for translocating enzymatic components of clostridial binary toxins. The findings that: (1) Iota toxicity was not inhibited by monensin alone; (2) a combination of monensin and valinomycin partially inhibited the toxin; and (3) monensin synergized the inhibitory effects of Baf, altogether strongly suggest that Iota toxin requires both proton and membrane potential gradients for Ia translocation into the cytosol, in a similar mechanism that was recently discovered for the eukaryotic cell growth factor, FGF-1 [35]. This current study is the first to report that a membrane potential gradient can also facilitate translocation of any bacterial toxin into the cytosol, and perhaps further investigation with other bacterial toxins may reveal similar exploitation of this inherent eukaryotic system by pathogens.

The effects of Iota and C2 toxin were not prevented by BFA and nocodazole. BFA inhibits the transport of ricin and Shiga toxins from endosomes to the Golgi apparatus [49], and nocodazole perturbs transport between early and late endosomes as well as movement from the Golgi apparatus to the ER [50]. These results are in agreement with a recent report [15], and an earlier finding [45] that there was no effect of *C. spiroforme* toxin when cells were co-incubated at 15 °C. This temperature prevents protein trafficking from endosomes to the trans Golgi network.

Two main types of intracellular routes for bacterial toxins are evident for translocating enzymatic domains into the cytosol. These routes include a long retrograde pathway from the plasma membrane to the ER through the Golgi apparatus (i.e. CT, *Pseudomonas* exotoxin A, ricin, and Shiga toxin), and a shorter route involving the endosomes (i.e. DT) [9]. Therefore, the results with pharmacological drugs and analysis of endocytosis of fluorescently labeled toxins indicate that Iota and C2 toxins use a short pathway that involves early, or possibly late, endosomes. The experiment where Baf was added at different times after Iota or C2 toxin (Fig. 4A) suggests that the endocytic process is shorter for C2, than for Iota toxin. C2 toxin could use a more efficient translocation mechanism than Iota, or each toxin could follow a different endocytic pathway after internalization from the plasma membrane.

In addition to the known effect of Baf as a specific blocker of vacuolar-ATPase and endosomal acidification, other effects of this drug include preventing the formation of intermediate vesicles (endocytic carrier vesicles, ECVs) from early endosomes [50,51], as well as inhibiting transport from late endosomes

to lysosomes [52,53]. Baf, which inhibited both Iota and C2 toxin effects, could therefore act by preventing endosomal acidification required for the translocation step as well as block endocytic transport of these toxins. It has been reported that in HeLa cells, Baf prevents the transport of rhinoviruses from early endosomes to ECVs, whereas nocodazole inhibits further trafficking from ECVs to late endosomes [54]. As shown with fluorescent Ib or C2-II, Baf as well as valinomycin, when given either alone or in combination with monensin, did not impair entry of these binding components from the plasma membrane to endocytic compartments. However, it cannot be ruled out that Baf influences trafficking within intracellular compartments. Since it takes more time for Iota toxin delivery into early endosomes versus C2 toxin (Fig. 4), it is likely that Iota toxin is translocated from ECVs because its cytopathic effects were blocked by Baf, but not nocodazole. ECVs are more acidic compartments (pH < 5.6) than early endosomes (pH 6.5) [54,55], and this pH difference could account for the inability of inhibitors against endosomal acidification to affect Iota cytotoxicity, in contrast to that of C2. The additive inhibitory effects of Baf and monensin also support the paradigm that Iota toxin translocates from vesicles, such as ECVs, which lack Na^+/K^+-ATPase [36].

Results from this study, as well as those from others, now provide a clearer picture for internalization and intracellular transport of Iota and C2 toxins (Fig. 8). Binding components of Iota and C2 toxins recognize specific cell-surface receptors, oligomerize into heptameric ring structures, form transmembrane channels, and dock with an enzymatic component(s) [14,15,26]. After endocytosis, the catalytic components escape the endosomal network to access the pool of cytosolic actin. While C2 toxin delivers its enzymatic component into the cytosol from early endosomes, Iota toxin further migrates until it reaches ECVs. Enzymatic components probably pass through the channel formed by oligomeric binding components following a pH gradient [30], and in addition a membrane potential gradient is uniquely required for translocating Iota toxin. The necessity of a membrane potential gradient for Ia translocation into the cytosol is a novel concept for any bacterial toxin, and it is likely that further studies will reveal similar mechanisms with other intracellular-acting toxins. This certainly does not preclude that other cytosolic factors are not also involved in translocating enzymatic toxin components into the cytosol, as shown recently for DT [56]. Indeed, heat shock protein 90 (Hsp-90) reportedly facilitates transport of C2-I and Ia, as well as FGF-1, across the endosomal membrane [57–59].

In summary, our study combines cell biology and drug-based experiments to provide a rather unique way of characterizing the trafficking-related molecular machinery involved in the onset of Iota and C2 cytotoxicity. While translocation of both toxins requires a low pH, only Iota toxin additionally employs a membrane potential, that also facilitates FGF-1

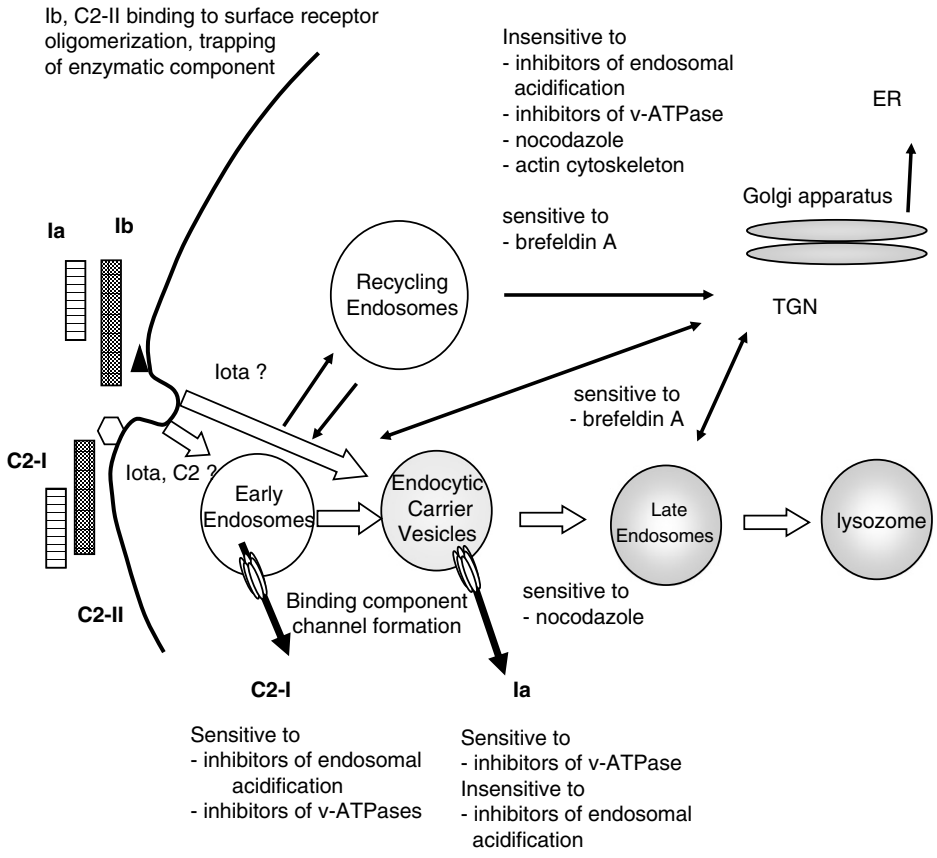


Fig. 8. A model for the endocytosis and translocation of Iota and C2 toxins. Binding components of Iota and C2 toxins recognize specific and distinct cell-surface receptors. Oligomerization of the binding components promotes transmembrane channel formation. The enzymatic component of C2 toxin is translocated from early endosomes into the cytosol by means of a pH gradient, likely via a channel formed by the binding component. Iota toxin, which requires more acidic conditions and longer time for internalization than C2 toxin, probably delivers its enzymatic component into the cytosol from endocytic carrier vesicles.

transport across endosomal membranes into the cytosol [35]. Studies focused upon these clostridial toxins indeed provide a further understanding of eukaryotic methods employed for transporting molecules across biological membranes, and such knowledge should facilitate more rational designs of delivery systems for therapeutic purposes.

Acknowledgement: This work was supported by Institut Pasteur funding (Grand Programme Horizontal no. 9).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.02.041.

References

[1] Gibert, M., Petit, L., Raffestin, S., Okabe, A. and Popoff, M.R. (2000) *Clostridium perfringens* iota-toxin requires activation of both binding and enzymatic components for cytopathic activity. Infect. Immun. 68, 3848–3853.
[2] Aktories, K., Bärmann, M., Ohishi, I., Tsuyama, S., Jakobs, K.H. and Habermann, E. (1986) Botulinum C2 toxin ADP-ribosylates actin. Nature 322, 390–392.
[3] Mauss, S., Chaponnier, C., Just, I., Aktories, K. and Gabbiani, G. (1990) ADP-ribosylation of actin isoforms by *Clostridium botulinum* C2 toxin and *Clostridium perfringens* iota toxin. Eur. J. Biochem. 194, 237–241.
[4] Popoff, M.R. and Boquet, P. (1988) *Clostridium spiroforme* toxin is a binary toxin which ADP-ribosylates cellular actin. Biochem. Biophys. Res. Commun. 152, 1361–1368.
[5] Considine, R.V. and Simpson, L.L. (1991) Cellular and molecular actions of binary toxins possessing ADP-ribosyltransferase activity. Toxicon 29, 913–936.
[6] Abrami, L., Liu, S., Cosson, P., Leppla, S.H. and van der Goot, F.G. (2003) Anthrax toxin triggers endocytosis of its receptor via a lipid raft-mediated clathrin-dependent process. J. Cell Biol. 160, 321–328.
[7] Leppla, S.H. (2000) in: Bacterial Protein Toxins (Aktories, K. and Just, I., Eds.), pp. 445–472, Springer, Berlin.
[8] Lamaze, C. and Johannes, L. (2006) in: The Sourcebook of Bacterial Protein Toxins (Alouf, J.E. and Popoff, M.R., Eds.), pp. 135–153, Elsevier Academic Press, Amsterdam.
[9] Olsnes, S., Wesche, J. and Falnes, P.O. (1999) in: The Comprehensive Sourcebook of Bacterial Protein Toxins (Alouf, J.E. and Freer, J.H., Eds.), pp. 73–93, Academic Press, London.
[10] Montecucco, C. (1998) Protein toxins and membrane transport. Curr. Opin. Cell Biol. 100, 530–536.
[11] Falnes, P.O. and Sandvig, K. (2000) Penetration of protein toxins into cells. Curr. Opin Cell Biol. 12, 407–413.
[12] Johannes, L. and Goud, B. (1998) Surfing on a retrograde wave: how does Shiga toxin reach the endoplasmic reticulum? Trends Cell Biol. 8, 158–162.
[13] Simpson, L.L. (1989) The binary toxin produced by *Clostridium botulinum* enters cells by receptor-mediated endocytosis to exert its pharmacologic effects. J. Pharmacol. Exp. Ther. 251, 1223–1228.
[14] Barth, H., Blöcker, D., Behlke, J., Bergsma-Schutter, W., Brisson, A., Benz, R. and Aktories, K. (2000) Cellular uptake of *Clostridium botulinum* C2 toxin requires oligomerization and acidification. J. Biol. Chem. 275, 18704–18711.
[15] Blöcker, D., Behlke, J., Aktories and Barth, H. (2001) Cellular uptake of the *Clostridium perfringens* binary iota-toxin. Infect. Immun. 69, 2980–2987.
[16] Perelle, S., Scalzo, S., Kochi, S., Mock, M. and Popoff, M.R. (1997) Immunological and functional comparison between *Clostridium perfringens* iota toxin, *C. spiroforme* toxin, and anthrax toxins. FEMS Microbiol. Lett. 146, 117–121.
[17] Knapp, O., Benz, R., Gibert, M., Marvaud, J.C. and Popoff, M.R. (2002) Interaction of *Clostridium perfringens* iota-toxin with lipid bilayer membranes. J. Biol. Chem. 277, 6143–6152.
[18] Koch, G., Benz, C., Schmidt, G., Olenik, C. and Aktories, K. (1997) Role of Rho protein in lovastatin-induced breakdown of actin cytoskeleton. J. Pharmacol. Exper. Ther. 283, 901–909.
[19] Machesky, L.M. and Hall, A. (1997) Role of actin polymerization and adhesion to extracellular matrix in Rac- and Rho-induced cytoskeletal reorganization. J. Cell Biol. 138, 913–926.
[20] Sandvig, K. and Olsnes, S. (1980) Diphtheria toxin entry into cells is falicitated by low pH. J. Cell Biol. 87, 828–832.
[21] Lemichez, E., Bomsel, M., Devilliers, G., vanderSpek, J., Murphy, J.R., Lukianov, E.V., Olsnes, S. and Boquet, P. (1997) Membrane translocation of diphtheria toxin fragment A exploits early to late endosome trafficking machinery. Mol. Microbiol. 23, 445–457.
[22] Hale, M., Marvaud, J.C., Popoff, M.R. and Stiles, B.G. (2004) Detergent-resistant membrane microdomains facilitate Ib oligomer formation and biological activity of *Clostridium perfringens* iota-toxin. Infect. Immun. 72, 2186–2193.
[23] Stiles, B.G., Hale, M.L., Marvaud, J.C. and Popoff, M.R. (2002) *Clostridium perfringens* iota toxin: characterization of the cell-asociated iota b complex. Biochem. J. 367, 801–808.
[24] Ohishi, I. and Yanagimoto, A. (1992) Visualizations of binding and internalization of two nonlinked protein components of botulinum C2 toxin in tissue culture cells. Infect. Immun. 60, 4648–4655.
[25] Richard, J.F., Mainguy, G., Gibert, M., Marvaud, J.C., Stiles, B.G. and Popoff, M.R. (2002) Transcytosis of iota toxin across polarized CaCo-2 cell monolayers. Mol. Microbiol. 43, 907–917.
[26] Stiles, B., Hale, M.L., Marvaud, J.C. and Popoff, M.R. (2000) *Clostridium perfringens* iota toxin: binding studies and characterization of cell surface receptor by fluorescence-activated cytometry. Infect. Immun. 68, 3475–3484.
[27] Stiles, B.G., Blöcker, D., Hale, M.L., Guetthoff, M.A. and Barth, H. (2002) *Clostridium botulinum* C2 toxin: binding studies with fluorescence-activated cytometry. Toxicon 40, 1135–1140.
[28] Sandvig, K. and van Deurs, B. (1994) Endocytosis and intracellular sorting of ricin and Shiga toxin. FEBS Lett. 346, 99–102.
[29] Smith, D.C., Lord, J.M., Roberts, L.M., Tartour, E. and Johannes, L. (2002) 1st class ticket to class I: protein toxins as pathfinders for antigen presentation. Traffic 3, 697–704.
[30] Bachmeyer, C., Benz, R., Barth, H., Aktories, K., Gibert, M. and Popoff, M.R. (2001) Interaction of *Clostridium botulinum* C2 toxin with lipid bilayer membranes and Vero cells: inhibition of channel function in chloroquine and related compounds in vitro and toxin action in vivo. FASEB J. 15, 1658–1660.
[31] Mellman, I., Fuchs, R. and Helenius, A. (1986) Acidification of the endocytic and exocytic pathways. Ann. Rev. Biochem. 55, 663–700.
[32] Bowman, E.J., Siebers, A. and Altendorf, K. (1988) Bafilomycins, a class of inhibitors of membrane ATPases from microorganisms, animal cells, and plant cells. Proc. Natl. Acad. Sci. USA 85, 7972–7976.
[33] Blöcker, D., Pohlmann, K., Haug, G., Bachmeyer, C., Benz, R., Aktories, K. and Barth, H. (2003) *Clostridium botulinum* C2 Toxin: low pH-induced pore formation is required for translocation of the enzyme component C2I into the cytosol of host cells. J. Biol. Chem. 278, 37360–37367.
[34] Yoshida, T., Chen, C., Zhang, M. and Wu, H.C. (1991) Disruption of the Golgi apparatus by brefeldin A inhibits the cytotoxcicity of ricin, modeccin, and *Pseudomonas* toxin. Exp. Cell Res. 192, 389–395.
[35] Malecki, J., Wiedlocha, A., Wesche, J. and Olsnes, S. (2002) Vesicle transmembrane potential is required for translocation to the cytosol of externally added FGF-1. EMBO J. 21, 4480–4490.
[36] Fuchs, R., Schmid, S. and Mellman, I. (1989) A possible role for Na+/K+-ATPase in regulating ATP-dependent endosome acidification. Proc. Natl. Acad. Sci (USA) 86, 539–543.
[37] Perelle, S., Gibert, M., Boquet, P. and Popoff, M.R. (1993) Characterization of *Clostridium perfringens* iota-toxin genes and expression in *Escherichia coli*. Infect. Immun. 61, 5147–5156, Author's correction, 63:4967, 1995.
[38] Lacy, B., Wigelsworth, D.J., Melnyk, R.A., Harrison, S.C. and Collier, R.J. (2004) Structure of heptameric protective antigen bound to an anthrax toxin receptor: a role for receptor in pH-dependent pore formation. Proc. Natl. Acad. Sci. USA 101, 13147–13151.

[39] Miller, C.J., Ellliot, J.L. and Collier, R.J. (1999) Anthrax protective antigen: prepore-to-pore conversion. Biochemistry 38, 10432–10441.
[40] Mogridge, J., Mourez, M. and Collier, R.J. (2001) Involvement of domain 3 on oligomerization by the protective antigen moiety of anthrax toxin. J. Bacteriol. 183, 2111–2116.
[41] Abrami, L., Lindsay, M., Parton, R.G., Leppla, S.H. and van der Goot, F.G. (2004) Membrane insertion of anthrax protective antigen and cytoplasmic delivery of lethal factor occur at different stages of the endocytic pathway. J. Cell Biol. 166, 645–651.
[42] Friedlander, A.M. (1986) Macrophages are sensititve to anthrax lethal toxin through an acid-dependent proces. J. Biol. Chem. 261, 7123–7126.
[43] Guidi-Rontani, C., Weber-Levy, M., Mock, M. and Cabiaux, V. (2000) Translocation of *Bacillus anthracis* lethal and edema factors across endosome membranes. Cell. Microbiol. 2, 259–264.
[44] Gibert, M., Perelle, S., Daube, G. and Popoff, M.R. (1997) *Clostridium spiroforme* toxin genes are related to *C. perfringens* iota toxin genes but have a different genomic localization. Syst. Appl. Microbiol. 20, 337–347.
[45] Popoff, M.R., Milward, F.W., Bancillon, B. and Boquet, P. (1989) Purification of the *Clostridium spiroforme* binary toxin and activity of the toxin on HEp-2 cells. Infect. Immun. 57, 2462–2469.
[46] Blöcker, D., Bachmeyer, C., Benz, R., Aktories, K. and Barth, H. (2003) Channel formation by the binding component of *Clostridium botulinum* C2 toxin: glutamate 307 of C2II affects channel properties in vitro and pH-dependent C2I translocation in vivo. Biochemistry 42, 5368–5377.
[47] Bachmeyer, C., Orlik, F., Barth, H., Aktories, K. and Benz, R. (2003) Mechanism of C2-toxin inhibition by Fluphenazine and related compounds: investigation of their binding kinetics to the C2II-channel using the current noise analysis. J. Mol. Biol. 333, 527–540.
[48] Blaustein, R.O. and Finkelstein, A. (1990) Voltage-dependent block of anthrax toxin channels in planar phospholipid bilayer membranes by symetric tetraalkylammonium ions. J. Gen. Physiol. 96, 905–919.
[49] Sandvig, K. and Van Deurs, B. (1996) Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. Physiol. Rev. 76, 949–966.
[50] Gruenberg, J. and Maxfield, F.R. (1995) Membrane transport in the endocytic pathway. Curr. Opin. Cell Biol. 7, 552–563.
[51] Clague, M.J., Urbel, S., Aniento, F. and Gruenberg, J. (1994) Vacuolar ATPase activity is required for endosomal carrier vesicle formation. J. Biol. Chem. 269, 21–24.
[52] van Weert, A.W., Dunn, K.W., Geuze, H.J. and Maxfield, F.R. (1995) Transport from late endosome to lysosomes, but not sorting of integral proteins in endosomes, depends on the vacuolar proton pump. J. Cell Biol. 130, 821–834.
[53] van Deurs, B., Holm, P.K. and Sandvig, K. (1996) Inhibition of the vesicular H+-ATPase with bafilomycin reduces delivery of internalized molecules from mature multivesicular endosomes to lysosomes in HEp-2 cells. Eur. J. Cell Biol. 69, 343–350.
[54] Bayer, N., Schober, D., Prchla, E., Murphy, R.F., Blaas, D. and Fuchs, R. (1998) Effect of bafilomycin A1 and nocodazole on endocytic transport in Hela cells: implication for viral uncoating and infection. J. Virol., 9645–9655.
[55] Mellman, I. (1996) Endocytosis and molecular sorting. Annu. Rev. Cell Dev. Biol. 12, 575–625.
[56] Ratts, R., Zeng, H., Berg, E.A., Blue, C., McCom, M.E., Costello, C.E., Vanderspek, J.C. and Murphy, J.R. (2003) The cytosolic entry of diphtheria toxin catalytic domain requires a host cell cytosolic translocation factor complex. J. Cell Biol. 160, 1139–1150.
[57] Haug, G., Leemhuis, J., Tiemann, D., Meyer, D.K., Aktories, K. and Barth, H. (2003) The host cell chaperonne Hsp90 is essential for translocation of the binary *Clostridium botulinum* C2 toxin into the cytosol. J. Biol. Chem. 278, 32266–32274.
[58] Hang, G., Aktories, K. and Barth, H. (2004) The host cell chaperone Hsp90 is necessary for cytotxic action of the binary iota-like toxins. Infect. Immun. 72, 3066–3068.
[59] Wesche, J., Malecki, J., Wiedlocha, A., Skjerpen, C.S., Claus, P. and Olsnes, S. (2006) FGF-1 and FGF-2 require the cytosolic chaperone Hsp90 for translocation into the cytosol and the cell nucleus. J. Biol. Chem. 281, 11405–11412.