The binary *Clostridium botulinum* C2 toxin consists of two individual proteins, the transport component C2II (80 kDa) and the enzyme component C2I, which ADP-ribosylates G-actin in the cytosol of cells. Trypsin-activated C2II (C2IIa) forms heptamers that bind to the cell receptor and mediate translocation of C2I from acidic endosomes into the cytosol of target cells. Here, we report that translocation of C2I across cell membranes is accompanied by pore formation of C2IIa. We used a radioactive rubidium release assay to detect C2IIa pores in the membranes of Chinese hamster ovary cells. Pore formation by C2IIa was dependent on the cellular C2 toxin receptor and an acidic pulse. Pores were formed when C2IIa was bound to cells at neutral pH and when cells were subsequently shifted to acidic medium (pH ≤ 5.5), but no pores were detected when C2IIa was added to cells directly in acidic medium. Most likely, acidification induces a change from “pre-pore” to “pore” conformation of C2IIa, and formation of the pore conformation before membrane binding precludes insertion into membranes. When C2I was present during binding of C2IIa to cells prior to the acidification step, C2IIa-mediated rubidium release was decreased, suggesting that C2I interacted with the lumen of the C2IIa pore. A decrease of rubidium efflux was also detected when C2I was added to C2IIa-treated cells after the acidification step, suggesting that C2I interacted with C2IIa in its pore conformation. Moreover, C2I also interacted with C2IIa channels in artificial lipid membranes and blocked them partially. C2I was only translocated across the cell membrane when C2IIa plus C2I were bound to cells at neutral pH and subsequently shifted to acidic pH. When cell-bound C2IIa was exposed to acidic pH prior to C2I addition, only residual intoxication of cells was observed at high toxin concentrations, and binding of C2I to C2IIa was slightly decreased. Overall, C2IIa pores were essential but not sufficient for translocation of C2I. Intoxication of target cells with C2 toxin requires a strictly coordinated pH-dependent sequence of binding, pore formation by C2IIa, and translocation of C2I.

Bacterial protein toxins, which target substrates in the cytosol of eukaryotic cells, have developed sophisticated delivery mechanisms to transport their active components across lipid membranes into the cytosol. Bacterial AB-type exotoxins consist of a binding (B-) domain, which binds to a specific receptor on target cells and mediates translocation of the enzymatic (A-) domain into the cytosol (1). Binary toxins are special type AB toxins, which have individually separated enzyme and binding components that have to assemble on the surface of target cells to act cytotoxic (for review, see Ref. 2). Lethal toxin and edema toxin from *Bacillus anthracis* (3) belong to this family as well as actin-ADP-ribosylating toxins. Actin-ADP-ribosylating toxins are *Clostridium botulinum* C2 toxin (4), iota toxin from *Clostridium perfringens* (5), ADP-ribosyltransferase (CDT) from *Clostridium difficile* (6), *Clostridium spiroforme* toxin (7, 8), and the vegetative insecticidal proteins (VIP) from *Bacillus cereus* (9). All these toxins modify G-actin at arginine 177 (4, 9). This causes depolymerization of actin filaments, breakdown of the actin cytoskeleton, and the rounding up of target cells (10, 11). The binding component of anthrax toxins, protective antigen (PA), shares homology with the binding components of actin-ADP-ribosylating toxins (1). PA forms heptameric pores in cell membranes under acidic conditions (12, 13), which are thought to be involved in translocation of the enzyme components (14).

Recently, we reported on the cellular uptake mechanism of *Clostridium botulinum* C2 toxin (15). C2 toxin consists of the enzyme component C2I (16) and the binding component C2II (17). Like PA, C2I has to be activated by proteolytic cleavage (18), and the resulting C2II forms ring-shaped heptamers (~420 kDa), which have an outer diameter of ~15 nm and an inner diameter of ~1–2 nm (15). C2I binds to its receptor on target cells (19). C2I assembles with C2IIa, and the complex is taken up by receptor-mediated endocytosis (20). C2I translocates from early acidic endosomes into the cytosol, a process that can be blocked by bafilomycin A1, a specific inhibitor of the vesicular H+ -ATPase (15). C2I is directly transported across the cell membrane via C2IIa when endosomal conditions are mimicked by extracellular acidification (15). However, the role of the C2IIa heptamer in the translocation of C2I across membranes is still not clear.

Because C2IIa forms channels in artificial lipid bilayer membranes (21), we addressed the question of whether C2IIa also forms pores in membranes of intact cells and whether these pores are involved in translocation of the enzyme component C2I into the cytosol of target cells. We found a pH-dependent
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Pore formation by C2Ia in cell membranes and showed that C2I interacted with C2Ia pores in vivo and in vitro. Taken together, translocation of C2I into the cytosol was only observed under conditions that induced pore formation of C2Ia in cell membranes and when both C2I and C2Ia were already bound to cells at the time of acidification.

EXPERIMENTAL PROCEDURES

Materials—The glutathione S-transferase gene fusion system and glutathione-Sepharose 4B were from Amersham Biosciences. Cell culture medium was purchased from Biochrom (Berlin, Germany), and fetal calf serum was obtained from PAN Systems (Aidenbach, Germany). Trypsinogen was obtained from Sigma. Trypsin, trypsin inhibitor, and the inhibitor were from Roche Diagnostics. Bafilomycin was from Calbiochem. Hanks’ balanced salt solution (HBSS) contained 0.185 g/liter CaCl2, 0.089 g/liter MgSO4, 0.4 g/liter KCl, 0.06 g/liter KH2PO4, 8 g/liter NaCl, 0.048 g/liter Na2HPO4, and 1 g/liter glucose to which 10 mM HEPES (pH 7.4) was added. Phosphate-buffered saline (PBS) contained 8 g/liter NaCl, 0.2 g/liter KCl, 1.15 g/liter Na2HPO4·2H2O, and 0.2 g/liter KH2PO4 (pH 7.4). Pierce IODO-BEADS were used for labeling C2I-S16C and C2Ia were incubated with Alexa488 at room temperature according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Excess of free Alexa488 was removed by washing with PBS in a Microcon 10 concentrator. Labeled proteins were stored at 4 °C (protected from light) and used within 2 weeks. Biological agents were of analytical grade and were purchased from commercial sources. Rubidium-86 was from PerkinElmer Life Sciences.

Expression, Purification, and Labeling of Proteins with Alexa488 and Na125I—Recombinant C2 proteins were expressed as glutathione S-transferase fusion proteins in Escherichia coli BL21 cells and purified as described previously (15, 22). C2I was activated with 0.2 μg of trypsin per microgram of protein for 30 min at 37 °C. Because C2I wild type does not provide a linkage site for Alexa488, C2I-S16C was used for labeling. C2I-S16C and C2Ia were incubated with Alexa488 at room temperature according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Excess of free Alexa488 was removed by washing with PBS in a Microcon 10 concentrator. Labeled proteins were stored at 4 °C (protected from light) and used within 2 weeks. Biological activity of labeled proteins was analyzed as described previously (23). Iodination of C2I was performed using IODO-BEADS according to the manufacturer’s (Pierce) protocol using 100 μCi of Na125I per 100 μg of C2I. In vivo activity of 125I-C2I was checked by incubation of cells with 125I-C2I together with C2Ia.

Cell Culture and Cytotoxicity Assays—All cells were cultured at 37 °C and 5% CO2, and were routinely trypsinized and reseded three times a week. CHO cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM/Hams’ F12 (1:1), containing 5% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Vero cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM), 100 units/ml penicillin, and 100 μg/ml streptomycin. Vero cells were maintained in DMEM supplemented with 10% fetal calf serum and antibiotics as described above. For cytotoxicity assays, cells were grown as subconfluent monolayers and treated with different concentrations of C2I toxins and the respective drug in serum-free medium.

Binding Experiments with Fluorescence-Activated Cytometry—Cells were detached from culture dishes with 50 mM EDTA in HBSS lacking Ca2+/Mg2+ and washed with ice-cold HBSS containing 0.2% bovine serum albumin (BSA). One million cells per tube were pelleted by centrifugation (800 × g) and resuspended in 50 μl of HBSS or medium. Various concentrations of C2I and/or C2Ia were added to cells and incubated at 4 °C for 20 min. For extracellular acidification experiments, cells were incubated with medium at pH 7.5 and 4.8, respectively, for 5 min at 37 °C. Thereafter, cells were washed three times with ice-cold HBSS-BSA and analyzed with a FACSCalibur flow cytometer (BD Biosciences).

Binding Experiments with Radiolabeled C2I—Vero cells (12-well plate) were incubated with serum-free DMEM containing 500 ng/ml C2I at 4 °C for 2 h to allow binding of C2Ia on the cell surface. Cells were washed two times with 1 ml of cold PBS per well. A pH shift was performed. Serum-free DMEM at pH 4.5 and 7.5 (3 ml/well), respectively, was added, and cells were kept at 37 °C and 5% CO2, for 5 min. Cells were washed twice with cold PBS and then incubated at 4 °C for 2 h with serum-free DMEM, pH 7.5, containing 400 ng/ml 125I-C2I for the binding of 125I-C2I to C2Ia. After two washing steps with cold PBS, cells were collected in 40 μl of boiling Laemmli sample buffer. 15 μl of cell lysate were subjected to 12.5% SDS-PAGE, and 125I-C2I was detected with a PhosphorImager from Amersham Biosciences.

Titration Experiments—[86Rb] Efflux Measurements—[86Rb] Efflux experiments were performed as described previously (24). CHO cells were plated in 24-well culture plates. 6 h after plating, fresh medium containing 1 Ci/ml of [86Rb] was added, and cells were incubated for a further 18 h. C2I proteins were added in a serum-free medium, and cells were incubated on ice for 1 h to allow toxin binding. Subsequently, the cells were washed two times with cold medium to remove unbound toxin. To initiate membrane insertion of the toxin, cells were treated with serum-free medium (pH 4.5–7.5) for 5 min at 37 °C. Cells were further incubated at 4 °C, and after 60 min the medium was removed and 86Rb release was determined by liquid scintillation counting.

Black Lipid Bilayer Experiments—Black lipid bilayer membranes were formed as described previously (25). The instrumentation consisted of a Teflon chamber with two aqueous compartments connected by a small circular hole with a surface area of ~0.5 mm2. Membranes were formed across the hole by painting onto a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster AL) in n-decane. The single channel recordings were performed using silver/silver chloride (with salt bridges) connected in a series to a voltage source and a current amplifier. The amplified signal was monitored on a storage oscilloscope (Tektronix 7633) and recorded on a strip chart or tape recorder. The temperature was maintained at 20 °C during all experiments. Titration measurements were performed with membranes containing 100–500 C2IIa channels as described earlier (21).

RESULTS

Activated C2IIa Forms Pores in Membranes of Intact Cells—We wanted to test whether C2IIa forms pores in cell membranes of CHO cells. Therefore, we used a radioactive assay to detect the release of 86Rb+ from preloaded cells (24). We found that treatment of CHO cells with trypsin-activated C2IIa resulted in pore formation in cell membranes under acidic conditions. For these experiments, C2IIa was bound to CHO monolayer cells at 4 °C and, after a pulse with acidic medium (37 °C), the released 86Rb+ was determined in the medium by scintillation counting. The morphology of CHO cells was not altered under these conditions. An acidic pulse (pH ≤ 5.5) was necessary to induce C2IIa pore formation (Fig. 1). Non-activated C2II did not form pores in membranes of intact cells under the same conditions (data not shown). Pore formation was dependent on the interaction of C2IIa with its receptor. CHO wild type cells and C2 toxin receptor-deficient mutant CHO-RK14 cells (26) were tested for C2IIa-mediated rubidium release. Pore formation was only observed in CHO wild type cells (Fig. 1), suggesting that binding of C2IIa to its receptor was an essential prerequisite for pore formation and that pores did not result from nonspecific insertion of C2IIa into membranes.

We characterized the conditions leading to pore formation by C2IIa in CHO wild type cells in more detail. It was essential that C2IIa was bound to cells at neutral pH prior to acidification. When cells were incubated with C2IIa in medium at pH 5.2, no pore formation was observed (Fig. 2). We found that insertion and pore formation of C2IIa in cell membranes was not reversible. When C2IIa was bound to cells at 4 °C and pH 7.5, and, thereafter, cells were shifted for 5 min at 37 °C to pH 5.2 and then back to fresh medium with pH 7.5 at 4 °C, 86Rb+ was still released from these cells (Fig. 2). Taken together, pore formation in cell membranes by C2IIa did result from a well defined sequence of events. First, C2IIa heptamers had to be bound to cell receptors at neutral pH, and then an acidic shift led to a conformational change of C2IIa and to insertion into membranes and pore formation. Most likely, C2IIa heptamers underwent a non-reversible conformational change after acidification and insertion into the membrane as pores.
Interaction of C2I with the C2IIa Pore in Vivo and in Vitro—Next, we studied the role of C2IIa pores for translocation of the enzyme component C2I. If the C2IIa pore is directly involved in translocation of C2I across cell membranes, C2I should interact with the lumen of the C2IIa pore and decrease efflux of rubidium from cells. To test this, CHO cells were incubated with C2IIa together with C2I at 4 °C to allow binding and, subsequently, cells were shifted for 5 min to fresh medium (pH 7.5, 37 °C) to initiate pore formation. During this acidification step (pH 5.2, 37 °C), C2I was added to C2IIa-treated cells, and the cells were incubated at 4 °C for 10 min. The release of rubidium from cells after 10 min was decreased compared with C2IIa (Fig. 3; C2IIa → C2I, 10 min), but not as much as when C2I was present during cell binding and acidification (Fig. 3; C2IIa + C2I, 10 min). Fresh medium (pH 5.2) was added and analyzed for rubidium efflux from cells after another 20 min of incubation at 4 °C. The efflux of rubidium from cells was more decreased than after 10 min (Fig. 3; C2IIa → C2I, 30 min), but impairment was not as complete as that detected for C2IIa + C2I (i.e., when C2I together with C2II was allowed to bind to cells). Our results clearly demonstrate that C2I is able to interact with cell-bound C2IIa under acidic conditions and reduce its channel activity. Based on this result, we tested the interaction of C2I with C2IIa channels in artificial black lipid bilayer membranes.

Titration Experiments of C2IIa-mediated Multichannel Conductance with C2I—The addition of C2Ia to one or both sides of a diphytanoyl phosphatidylcholine/n-decane membrane resulted in a strong conductance increase of the lipid bilayer membranes within ~20–30 min (21, 27). When C2I (60 ng/ml) was added after this time (when no more channels were inserted into the membrane to the trans-side of the membrane, i.e., the side opposite to the addition of C2Ia), no influence on the C2IIa-mediated membrane conductance was observed (left-hand side arrow of Fig. 4). This feature did not change when C2I was added at a higher concentration (240 ng/ml) or when the membrane conductance was followed over a longer period of time (up to 20 min). However, a strong effect on membrane conductance was observed when C2I was added at the same concentration (60 ng/ml) to the cis-side (right-hand side arrow in Fig. 4). Immediately after the addition of C2I, the membrane conductance started to decrease and was diminished to ~30–40% of its initial value. Simultaneously, the current noise showed a dramatic increase, indicating rapid flickering of the C2IIa channels. Further addition of C2I did not result in a further decrease of the current. This effect may be explained by a binding of C2I to the C2IIa heptamers, thus partially blocking the translocation of ions through the channel. Obviously,
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Fig. 3. Effect of C2I on C2IIa pores in CHO cells. CHO cells were loaded with 86Rb+ (1 μCi/ml) and subsequently incubated in fresh medium for 2 h at 4 °C with either C2IIa (200 ng/ml) or C2IIa + C2I (2 μg/ml). Cells were shifted for 5 min at 37 °C to pH 5.2 (during this step, C2I (2 μg/ml) was added again). To some samples, which have been incubated with C2IIa, C2I (2 μg/ml) was added after the pH step was done. Cells were further incubated at 4 °C and pH 5.2. After 10 min, the complete supernatant was removed and measured for 86Rb+ by scintillation counting (10-min values). Fresh medium (pH 5.2) was added, and, after a further 20 min, the complete supernatant was measured for 86Rb+ (30-min values). Values are given as mean ± S.D. (n = 3).

Fig. 4. Effect of C2I on channels formed by C2IIa. The membrane was formed from diphytanoyl phosphatidylcholine/n-decane. The aqueous phase contained 150 mM NaCl, pH 6, and 20 ng/ml C2IIa was added to the cis-side of the membrane. 45 min after the addition of C2IIa, 60 ng/ml C2I were first added to the trans-side (left hand side arrow), which did not influence the membrane conductance. 12 min later, C2I (60 ng/ml) was added to the cis-side (right hand side arrow), which led to a strong decrease of membrane conductance. The temperature was 20 °C, and the applied voltage was 20 mV at the cis-side.

binding is only possible to the cis-side of the C2IIa heptamers but not to the trans side. This result represents a further argument for the oriented insertion of the C2IIa heptamers into lipid bilayers (27). It is noteworthy that the C2IIa channels are not completely closed under these conditions, because they still conduct ions when C2I is bound.

Single-channel Studies—The results described above suggest a blockage of the C2IIa channel by C2I. To study this phenomenon in more detail, we performed single-channel studies under similar conditions. For these experiments we mixed concentrated solutions of C2IIa (50–100 ng/μl) and C2I (100 ng/μl) in a ratio of 1:4 and incubated them for 12–24 h at 4 °C. When lipid bilayer membranes were in the black state, small aliquots (2–5 μl) of C2IIa pre-incubated with C2I were added to the 5-ml 1 M KCl solution at the cis-side of the membranes. Single channels occurred after some time. Fig. 5 shows a typical experiment observed under these conditions. The left-hand side arrow (Fig. 5) indicates the reconstitution of a C2IIa heptamer into the black lipid bilayer. After ~1 min the channel became blocked by its interaction with C2I (middle arrow; Fig. 5). After about another 1.5 min a second channel occurred, which was already blocked by a C2I molecule (right-hand side arrow; Fig. 5). Fig. 5 clearly indicates that the block of the C2IIa channel by C2I was not a permanent one. Instead, the channels showed partial closure and exhibited considerable current noise. On average, the single C2IIa showed closure by ~60%. Taken together, the results of the multi-channel titration experiments (Fig. 4) showed perfect agreement with the single-channel data. Both are consistent with partial closure of the C2IIa channel by C2I and confirm the finding of the in vivo experiments that C2I decreases C2IIa-mediated rubidium efflux from cells.

pH-dependent C2I Translocation into the Cytosol—C2 toxin enters the cytosol of target cells in a pH-dependent manner from an endosomal compartment (15). However, the precise mechanism of membrane translocation of C2I has not yet been described. Several models are currently discussed; either the channel, which is formed under acidic conditions, is capable of transporting several enzyme components into the cytosol, or the process of membrane insertion and subsequent channel formation is the driving force for membrane translocation of C2I. To elucidate the role of the acidic pH-dependent pore formation in C2I transport into the cytosol, we performed experiments in which endosomal conditions were mimicked by extracellular acidification (15). To block toxin uptake by its normal route via an endosomal compartment, we pretreated Vero cells with bafilomycin A1, an inhibitor of the vesicular H+-ATPase. The cells were pre-chilled and incubated with C2IIa or C2IIa plus C2I, respectively, for 20 min at 4 °C to allow toxin binding. Thereafter, cells were washed with ice-cold PBS and incubated for 5 min at 37 °C with prewarmed medium at pH 7.5 or 4.8, respectively. The cells were washed, fresh medium at pH 7.5 (with or without C2I) was added, and the cells were further incubated at 37 °C for 2 h. As shown in Fig. 6A, cells only exhibited C2 toxin morphology when both C2IIa and C2I were present at the time point of low pH medium
addition. In the absence of C2IIa, C2I did not induce cell round up under these conditions, i.e. C2IIa was absolutely essential for pH-induced delivery of C2I into cells across cell membranes. Cells to which C2I was added after the acidification step did not round up. However, although our rubidium efflux experiments indicate that the C2IIa pore is not closed at neutral pH, a pH gradient across the cell membrane might be a necessary pre-requisite for C2I translocation into the cytosol.

Therefore, we performed a second set of experiments in which we incubated the cells with acidic medium for 5 min as usual and then added C2I directly into the low pH medium and incubated the cells for a further 5 min. The cells were washed and incubated in pH 7.5 medium for 2 h. Again, cells did not round up when C2I was added after C2IIa had been exposed to the acidic pH medium (Fig. 6B). Cell round up was detected only when the cells were incubated with extremely high concentrations of C2IIa. As shown in Fig. 6C, the “low pH” treatment of cell-bound C2IIa resulted in a 50-fold reduction of cytotoxic activity.

To exclude the possibility that C2I was unfolded or inactivated by acidic pH, we incubated C2I with low pH medium for 5 min at 37 °C. When this preparation was added together with C2IIa to cells in pH 7.5 medium, the cells rounded up as usual, indicating that the low pH treatment had no influence on the biological activity of C2I (not shown). Taken together, these data show that under conditions at which pore formation occurs, C2I is only translocated into the cytosol when it is bound to C2IIa at the time point of acidic pH medium addition. These results indicate that pore formation most likely is necessary but not sufficient for C2I transport.

Finally, we analyzed the interaction between C2I and C2IIa after acidification with two different approaches. First, flow cytometry was used. CHO cells were incubated with C2IIa on ice to allow toxin binding. Thereafter, cells were washed and incubated for 2 min at 37 °C with pH 4.8 medium. Alexa488-labeled C2I was added, and cells were further incubated for 4 min at 37 °C. The cells were washed to remove unbound toxin, and the amount of cell-bound C2I was measured by flow cytometry. Binding of C2I to C2IIa was reduced by ~40–50% by the low pH treatment (not shown). Control experiments with Alexa488-labeled C2IIa demonstrated that the amount of cell-bound C2IIa was not reduced by the low pH treatment (not shown). In another experimental design, we studied binding of radiolabeled C2I to C2IIa after acidification. Therefore, C2IIa was allowed to bind to Vero cells at 4 °C in neutral medium, and, thereafter, cells were exposed to a short acidic pulse (pH 4.5, 37 °C). Finally, cells were incubated with 125I-C2I at 4 °C in neutral medium. For control, all steps were performed in neutral medium. Again, 125I-C2I was still able to bind to C2IIa when cell-bound C2IIa was exposed to acidic medium prior to the addition of 125I-C2I (data not shown). However, binding of C2I to acidified C2IIa was slightly reduced compared with binding of C2I to C2IIa under neutral conditions, but the reduction of C2I binding is only small compared with the reduction of cytotoxicity.

**Binding of C2IIa to Cells in Acidic pH Medium—** As described above, no pore formation was detectable in 86Rb+ efflux experiments when C2IIa was exposed to acidic pH prior to its addition to cells. Because C2IIa-dependent pore formation was only possible after the interaction of C2IIa with its cellular receptor, we were interested in testing whether acidic pH treatment of C2IIa resulted in an inhibition of receptor binding. For these experiments, Alexa488-labeled C2IIa was treated with medium at pH 4.8 for 5 min at 37 °C. This preparation was then added to pre-chilled CHO cells. The cells were incubated at 4 °C in medium at pH 4.8 for 20 min to allow toxin binding. Thereafter, cells were washed to remove unbound toxin, and the amount of cell-bound C2IIa was determined by flow cytometry. The acidic pH treatment reduced C2IIa-binding to cells by ~30–40% (Fig. 7). This result indicates that the low pH treatment had some influence on the C2IIa receptor interaction. However, in contrast to pore formation, receptor binding was only reduced but not completely abolished.

**Discussion**

Binary toxins are composed of two individual proteins. We used this advantage to study the role of the binding and translocation component C2II from the binary actin-ADP-ribosylating C. botulinum C2 toxin for the delivery of the enzyme component C2I into the cytosol of host cells. After proteolytic activation, C2IIa forms ring-shaped heptamers that bind to cell receptors (carbohydrate structures) and interact with the enzyme component C2I. In the next step, the receptor-toxin complex is endocytosed. We have shown previously that C2I translocates from acidic endosomes into the host cell cytosol (15). This process was mimicked at the cell surface when the extracellular medium was acidified to imitate the pH of the endosomal lumen. Under such conditions, cell-bound C2I was delivered in a C2IIa-dependent manner directly across the cell membrane into the cytosol by the acidic pulse (15). However, the precise role of C2IIa in the translocation of C2I across membranes is still enigmatic.

In this communication, we report that C2II forms pores in membranes of intact cells and artificial lipid bilayers. Pore formation in cell membranes was determined by the release of 86Rb+ ions from preloaded cells. The data support our recent model for the uptake of C2 toxin and allow several novel conclusions on the mechanisms underlying the translocation process of binary toxins in general. The major findings were as follows. First, pore formation was restricted to activated C2IIa, i.e. the heptameric ring-shaped form of C2II. Non-activated C2IIa was not capable of inducing 86Rb+ release. Second, simi-
Fig. 6. pH-dependent C2I translocation into the cytosol of Vero cells. A, panel 1, control cells incubated without any toxin; panel 2, cells that have been incubated for 3 h at 37 °C in the presence of C2IIa (200 ng/ml) and C2I (100 ng/ml); panels 3 and 4, bafilomycin A1-pretreated (100 nM) cells, which were incubated with C2IIa (200 ng/ml) and C2I (100 ng/ml) for 20 min at 4 °C in serum-free medium. Cells were washed and incubated for 5 min with medium at pH 7.5 or 4.8, respectively. Medium (pH 7.5) was added, and cells were further incubated at 37 °C. 5, Bafilomycin A1-pretreated cells were incubated with 200 ng/ml C2IIa for 20 min at 4 °C in serum-free medium. Cells were washed and incubated for 5 min with medium at pH 4.8. Thereafter, C2I was added, and cells were further incubated in pH 7.5 medium at 37 °C. After 2 h, phase-contrast pictures were taken. B, panel 1, control cells incubated without any toxin; panel 2, bafilomycin A1-pretreated cells incubated with C2IIa (200 ng/ml) for 20 min at 4 °C in serum-free medium. Cells were washed and incubated for 5 min with medium at pH 4.8. Thereafter, C2I was added, and cells were further incubated in pH 4.8 medium at 37 °C for 5 min. The medium was replaced by pH 7.5 medium, and the cells were incubated for an additional 2 h at 37 °C. C, Vero cells were incubated with the indicated amounts of C2IIa for 20 min at 4 °C to allow binding of C2IIa. Thereafter, cells were washed and incubated for 5 min with medium at pH 7.5 and 4.8, respectively. C2I (100 ng/ml) was added, and the cells were further incubated at 37 °C and pH 7.5. After 2 h, phase-contrast pictures were taken, and the percentage of cells rounded up was determined. Values are given as mean ± S.D. (n = 3).
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FIG. 7. Binding of C2IIa to cells at acidic pH. Alexa488-labeled C2IIa (8 μg/ml) was incubated in DMEM/Hams’ P12 (1:1) medium at pH 7.5 for 30 min at 37 °C for 5 min. Thereafter, this preparation was added to CHO cells, and cells were incubated for 20 min on ice to allow toxin binding. The cells were washed to remove unbound toxin, and the amount of cell-bound C2IIa was determined by flow cytometry using 10⁵ cells to provide a fluorescence profile.

larly as was recently observed for the intoxication process (15), the formation of pores depended exclusively on acidic pH (pH ≤ 5.5). Third, pore formation in intact cells by C2IIa was receptor-dependent and not due to nonspecific insertion of the heptamers into biological membranes. Accordingly, receptor-deficient cells (CHO-RK14 cells) did not release ⁸⁶Rb⁺ ions. Fourth, pore formation was only detectable when C2IIa was allowed to bind to cells prior to the acidic pulse. This finding suggests that cell-bound C2IIa changes from a “pre-pore” conformation (this term was used in analogy to anthrax toxin in Ref. 28; see below) to the “pore” conformation, which inserts into cell membranes. C2IIa, which was already brought to the pore conformation by a previous low pH treatment before it was applied to cells, did not form pores. Finally, we observed that C2I interacted with C2IIa pores in artificial lipid membranes as well as in cell membranes and provided evidence that in both models the channel activity was reduced by C2I. However, there exist also some differences, which are easy to understand. Channel formation by C2IIa in lipid bilayers are not dependent on the presence of a receptor, probably because the surface of the membranes is smooth and does not contain surface structures such as biological membranes that hinder the interaction between C2IIa and the membrane surface.

Altogether, our results suggest that the C2IIa pore is not just a passive channel in membranes through which C2I proteins reach the cytosol. Translocation of C2I through C2IIa pores rather depends on a well regulated sequence of events. The data obtained suggest the following model for cellular uptake of C2 toxin. After toxin activation, C2IIa forms a heptameric pore-like structure in solution, the so-called pre-pore conformation. Thereafter, the C2IIa pre-pore binds to its receptor on the cell surface and assembles with the C2I enzyme component. After receptor-mediated endocytosis, the receptor/toxin complex is transported within endosomal compartments. In line with previous reports on toxin uptake, we suggest that acidification of endosomes drives the insertion of hydrophobic C2IIa-domains into the membrane (pore conformation) and participates in C2I translocation across the endosomal membrane into the cytosol. Moreover, our data suggest that both events, membrane insertion of the C2IIa pore and translocation of C2I across the membrane, are somehow coupled. Thus, it was essential for acidic pulse-induced translocation that C2I was bound to C2IIa on cells under neutral conditions before the pH shift. When the C2IIa pores were already formed prior to the addition of C2I, no intoxication of cells was observed, even under acidic conditions. When both toxin components were applied to cells in acidic medium, no intoxication was achieved either. Therefore, pore formation by C2IIa, the interaction of C2IIa with C2I, and the translocation of C2I must occur in a concerted manner.

Our data indicate an interaction of C2I with the C2IIa-channel vestibule or lumen. However, we have no direct evidence that C2I translocates via the lumen of the pore across the membrane. Recently, Knapp et al. showed that Ib, the binding component of C. perfringens iota toxin and another member of the family of binary actin-ADP-ribosylating toxins, forms pores in black lipid bilayer membranes and that these pores are partially “closed” by the enzyme component Ia (29).

Similar findings as those observed for the C2 toxin were reported for the tripartite anthrax system. PA, which delivers either lethal factor or edema factor into cells, is the central protein of binary anthrax toxins (for review, see Ref. 30). Like C2IIa, heptameric PA₆₃ forms pores in artificial lipid bilayer membranes and membranes of intact cells (12, 31). Moreover, PA₆₃ and C2IIa show functional similarities concerning the conversion of the pre-pore to the pore conformation. For both PA₆₃ and C2IIa this step is strictly dependent on acidic conditions (28). When the heptameric pre-pore of PA₆₃ is exposed to acidic medium (pH ≤ 7), the ring-shaped pre-pore form is converted into the pore conformation (28). Similar to our observations with C2IIa, PA₆₃, which is in the pore conformation prior to its addition to cells, is able to bind to the cellular receptors, but it neither forms pores in membranes nor is it able to mediate translocation of the enzyme component (28). The role of the PA₆₃ pore in translocation of the anthrax enzyme components has been analyzed previously and discussed by Sellman et al. (14). The exchange of three amino acid residues in PA at positions 397, 425, and 427, which are located in the membrane insertion domain 2, blocked pore formation and translocation of PA ligands, although this exchange had no effect on receptor binding of PA (14). Domain 2 of PA contains large flexible loops, which are implicated in membrane insertion and pore formation. Therefore, pore formation of PA₆₃ appears to be essential for the delivery of cytotoxic PA ligands into the cytosol of host cells. The hypothesis of Miller et al. (28), i.e. that the conversion from the pre-pore to the pore conformation of PA₆₃ could be a first trigger for translocation of PA-bound enzyme components and their unfolding, is in agreement with our model for translocation of the C2 toxin described in this communication.

To our knowledge, it has never been shown for any toxin whether the A-domain is directly translocated through pores formed by the binding components. Therefore, one major question is whether C2I is translocated directly through the C2IIa pore. Considering the hypothesis that C2IIa pores have an inner diameter of −1–2 nm, which is based on electron microscopic studies (15), at least a partial unfolding of the C2I protein (50 kDa) should be expected, implying refolding of the toxin in the cytosol. This hypothesis is strengthened by our recent observation that a cellular chaperone most likely mediates translocation and/or refolding of C2I. We found that heat shock protein Hsp90 is essential for uptake of active C2I from endosomes into the cytosol of cells (32). If C2I has to become unfolded for its translocation through endosomal membranes,
one important question is how unfolding is achieved. Based on the present findings, we suggest that concerted binding of C2I to C2IIa and low pH are both essential to trigger this unfolding process.

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