Binding and Internalization of *Clostridium botulinum* C2 Toxin[▽]

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Clostridium botulinum C2 toxin is a binary toxin composed of an enzymatic component (C2I) and a binding component (C2II). The activated binding component (C2IIa) forms heptamers, and the oligomer with C2I is taken up by receptor-mediated endocytosis. We investigated the binding and internalization of C2IIa in cells. The C2IIa monomer formed oligomers on lipid rafts in membranes of MDCK cells. Methyl-beta-cyclodextrin inhibited the binding of C2IIa and the rounding of the cells induced by C2I plus C2IIa. C2I was localized to the rafts in the presence, but not the absence, of C2IIa. Surface plasmon resonance analysis revealed that C2I bound to the oligomer of C2IIa, but not the monomer of C2IIa. C2I and C2IIa were rapidly internalized in the cells. LY294002, a phosphatidylinositol 3-kinase (PI3K) inhibitor, inhibited the internalization of C2IIa in the cells and the rounding activity in the presence of C2I plus C2IIa. Incubation of the cells with C2I plus C2IIa resulted in the activation of PI3K and in phosphorylation of phosphoinositide-dependent kinase 1 and protein kinase B/Akt (Akt), but that with C2IIa alone did not. Akt inhibitor X, an Akt phosphorylation inhibitor, inhibited the rounding activity but not the internalization of C2IIa. The results suggest that the binding of C2I to the oligomer of C2IIa on rafts triggers the activation of the PI3K-Akt signaling pathway and, in turn, the initiation of endocytosis.

Clostridium botulinum produces botulinum C2 toxin by recruiting a binding component (C2II) to deliver the enzymatic component (C2I) to the interior of eukaryotic cells (4, 8). Each protein has been reported to lack toxic activity when injected alone (8). These proteins act in binary combinations to produce toxic, cytotoxic, and lethal effects, and they influence vascular permeability (8). The cleavage of C2II by trypsin removes the N-terminal 20-kDa fragment (C2IIa), thereby activating C2II (8). C2 toxin belongs to a family of binary actin-ADP-ribosylating toxins that includes *Clostridium perfringens* iota-toxin, *Clostridium spiroforme* iota-like toxin, *Clostridium difficile* ADP-ribosyltransferase, and vegetative insecticidal protein (VIP) from *Bacillus cereus* (8).

C2I ADP-ribosylates monomeric actin in the cytosol, at arginine-177 (3, 36). The ADP-ribosylation causes the breakdown of F-actin, leading to cell rounding and death. The crystal structure of C2I (29) shows that its closest structural relatives are the enzymatic components Iota-a (Ia) of iota-toxin (32, 33) and VIP2 of VIP (14). C2I has the same two-domain structure (N-terminal domain and C-terminal domain) as Ia and VIP2. The N- and C-terminal domains of the enzymatic component play a role in the interaction with the binding component and the catalytic function, respectively (6, 29). The amino acid sequence of C2II is similar to those of Ib of iota-toxin and the protective antigen (PA) of *Bacillus anthracis* (8). C2II is structurally similar to PA (29). C2II and PA are comprised of four domains. In PA, domain 1 is involved in inter-

action with the enzymatic component (lethal factor or edema factor), domain 2 in pore formation, domain 3 in oligomerization, and domain 4 in receptor binding. PA also forms ring-shaped heptamers, which are responsible for the delivery of the enzymatic component of anthrax toxin into the cytosol (8), suggesting that each domain of C2II has the same function as that of PA.

C2IIa recognizes the asparagine-linked carbohydrates on the surfaces of target cells and forms heptamers that bind C2I (11). The toxin-receptor complex is internalized by receptor-mediated endocytosis and translocated to the early endosomes (7). At the acidic pH of the endosomal compartment with vesicular H⁺-ATPase, the C2IIa oligomer is inserted into the membrane and forms pores, through which the bound C2I is then translocated into the cytosol (7). After translocation to the cytosol, refolding of the C2I protein is facilitated by the chaperone Hsp90 in the cytosol (15). C2I ADP-ribosylates G-actin in the cytosol. Subsequently, the event causes the depolymerization of actin filaments, breakdown of the actin cytoskeleton, and rounding of the cells (8). It has been reported that PA and Ib induce endocytosis of their receptors via a lipid raft-mediated process (2, 23). However, little is known about the binding to and initial entry into cells by C2 toxin. Here we present evidence for the binding of C2IIa to lipid rafts of MDCK cells and the internalization of C2IIa with C2I into the cells.

MATERIALS AND METHODS

Materials. Methyl-beta-cyclodextrin (MBCD), quercetin dihydrate, L- α -phosphatidylinositol, and a protease inhibitor mixture were obtained from Sigma (St. Louis, MO). LY294002, wortmannin, LY303511, and Akt inhibitor X were purchased from Calbiochem (San Diego, CA). Rabbit anti-phospho-Akt (Ser472), anti-Akt, anti-phospho-phosphoinositide-dependent kinase 1 (anti-phospho-PDK1), and anti-PDK1 antibodies were purchased from Cell Signaling (Danvers,

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MA). Mouse anti-caveolin-1 and Lyn antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phosphatidylinositol 3-kinase (anti-PI3K) p85 antibody was purchased from Upstate Biotechnology (Charlottesville, VA). Horseradish peroxidase-labeled anti-rabbit immunoglobulin G (IgG), horseradish peroxidase-labeled anti-mouse IgG, the pGEX4T-1 vector, protein G-Sepharose, and an enhanced chemiluminescence kit were obtained from GE Healthcare (Tokyo, Japan). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco BRL (New York, NY). Alexa Fluor 488-conjugated goat anti-rabbit IgG and rhodamine-phalloidin were obtained from Molecular Probes (Eugene, OR). Restriction endonucleases and DNA-modifying enzymes were obtained from Toyobo (Osaka, Japan). All other chemicals were of the highest grade available from commercial sources.

Expression and purification of C2I and C2II. The C2I and C2II genes from *C. botulinum* type C strain C203U28NT were amplified by PCR with chromosomal DNA, using the following primers: for C2I, primers C2I-01 (5'-GGAGATCTATGCCAATAATAAAAGAA-3'), containing a BglII site (in bold), and C2I-02 (5'-CCGTCGACCTAAATCTCTTTATT-3'), containing a SalI site (in bold); and for C2II, primers C2II-01 (5'-GCTTCGGGATCCATGTTAGTTTCAAAAATTGAG-3'), containing a BamHI site (in bold), and C2II-02 (5'-TCGATCCTCGAGCTATATTATTTATCTAA-3'), containing an XhoI site (in bold) (12, 18). The PCR products were cloned into the pT7Blue-T vector (Novagen, Madison, WI), resulting in the plasmids pT-C2I and pT-C2II. For expression experiments, the C2I gene and the C2II gene were excised with BglII/SalI and BamHI/XhoI, respectively, and cloned into BamHI/SalI-digested- and BamHI/XhoI-digested pGEX4T-1, resulting in pGEX4T-C2I and pGEX4T-C2II, respectively. Recombinant *C. botulinum* C2I and C2II were expressed in *Escherichia coli* as glutathione S-transferase fusion proteins, and the toxin components were liberated using thrombin as described by Barth et al. (7). C2II was activated by incubation with 0.2 µg of trypsin/µg of C2II at 37°C for 30 min. The reaction was stopped by adding a trypsin inhibitor (2 µg/µg of trypsin). C2I and C2II were stored at -20°C.

Preparation of anti-C2I and -C2II antisera. Antisera for C2I and C2II were prepared by immunizing rabbits with purified C2I and purified C2II according to a modification of the preparation method used to prepare anti-Ia and -Ib antisera (28). Freund incomplete adjuvant (Difco Laboratories, Detroit, MI) was mixed with C2I (100 µg) or C2II (100 µg) (1:1). Two intramuscular booster injections of the antigen were given. Antisera were obtained 1 week after the last injection.

Assay of cytotoxicity. MDCK cells were obtained from Riken Cell Bank (Tsukuba, Japan). They were cultured in DMEM supplemented with 10% fetal calf serum (FCS), 100 units/ml of penicillin, 100 µg/ml of streptomycin, and 2 mM glutamine (FCS-DMEM). All incubation steps were carried out at 37°C in a 5% CO₂ atmosphere.

The test for cytotoxicity was done on MDCK cells. The cells were cultivated in FCS-DMEM. For cytotoxicity assays, the cells were inoculated in 48-well tissue culture plates (Falcon, Oxnard, CA). Various concentrations of C2I and C2IIa were mixed in FCS-DMEM and inoculated onto cell monolayers. The cells were observed for morphological alterations 8 h after inoculation, as described previously (21). For cholesterol inhibition assays, C2IIa was preincubated with cholesterol (stock solution in ethanol; working concentration of cholesterol, 50 µg/ml) or a control (ethanol alone at the corresponding dilution) at 37°C for 30 min to use in the assay. The final ethanol concentration was 0.1%. To measure the effects of LY294002, quercetin, wortmannin, LY303511, and Akt inhibitor X on the cytotoxicity of C2 toxin, MDCK cells were preincubated with these agents at 37°C for 1 h and then incubated with C2I and C2IIa at 37°C for 8 h.

Procedure of iodination. ¹²⁵I-labeled C2I, C2II, and C2IIa were prepared with Bolton and Hunter reagent (2,000 Ci/mmol; GE Healthcare) as described previously (21). C2I, C2II, and C2IIa (50 µg) were incubated with 250 µCi of ¹²⁵I-labeled Bolton-Hunter reagent. Labeled C2I plus labeled C2IIa retained over 90% of the original toxicity (cytotoxicity) of C2I plus C2IIa.

Sucrose gradient fractionation. The separation of lipid rafts was carried out by flotation-centrifugation on a sucrose gradient (22, 23). MDCK cells were plated in 100-mm-diameter tissue culture dishes 24 h before use. ¹²⁵I-labeled C2IIa was added to cells in FCS-DMEM at 4°C for 1 h. The cells were washed and transferred into warmed FCS-DMEM (37°C) for various periods. The cells were rinsed with Hanks balanced salt solution (HBSS) and then lysed through exposure to 1% Triton X-100 at 4°C for 30 min in HBSS containing a protease inhibitor mixture. The lysates were scraped from the dishes with a cell scraper and homogenized by passage through a 22-gauge needle. The lysates were adjusted to 40% sucrose (wt/vol), overlaid with 2.4 ml of 36% sucrose and 1.2 ml of 5% sucrose in HBSS, centrifuged at 45,000 rpm (250,000 × g) at 4°C for 18 h in an SW55 rotor (Beckman Instruments, Inc., Palo Alto, CA), and fractionated

from the top (0.4 ml/fraction). The aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and autoradiographed.

Immunoblot analysis. For lipid raft marker proteins, aliquots of the flotation sucrose gradient fractions were heated in 2% SDS sample buffer at 99°C for 3 min. For detection of phosphorylation of Akt and PDK1, MDCK cells were incubated with C2I plus C2IIa or C2IIa only in FCS-DMEM at 37°C for various periods. After incubation, the reaction was terminated by the addition of 0.5 ml of ice-cold 7.5% trichloroacetic acid containing 0.1 mM Na₃VO₄ and kept on ice for 30 min. The precipitate was collected by centrifugation at 10,000 × g for 20 min and heated in 2% SDS sample buffer at 99°C for 3 min. The samples were electrophoresed in an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. The membrane was blocked with 20 mM Tris-HCl buffer (pH 7.5)-0.9% saline (TBS) containing 2% Tween 20 and 5% bovine serum albumin (BSA) and incubated first with the primary antibody in TBS containing 1% BSA, then with a horseradish peroxidase-conjugated secondary antibody, and finally with the reagents from an enhanced chemiluminescence analysis kit.

Cholesterol depletion. To remove cholesterol from MDCK cells, the cells were incubated at 37°C for 1 h in the presence of MβCD in HBSS and then washed with HBSS. Cholesterol levels were assayed spectrophotometrically using a diagnostic kit (cholesterol C-test; Wako Pure Chem, Osaka, Japan) (22).

Determination of molecular weight of C2IIa oligomer by SDS-3.5% PAGE. To determine the molecular weight of the C2IIa oligomer, SDS-PAGE with a 3.5% gel was performed according to a previously described method (21). High-molecular-mass standards for SDS-PAGE were phosphorylase b cross-linked SDS molecular size markers (Sigma), including monomers (97 kDa), dimers (194 kDa), trimers (292 kDa), tetramers (390 kDa), pentamers (487 kDa), and hexamers (584 kDa). Low-molecular-mass standards containing phosphorylase b (94 kDa), albumin (67 kDa), and ovalbumin (43 kDa) were obtained from GE Healthcare.

Immunofluorescence staining and confocal imaging. MDCK cells were plated on a polylysine-coated glass-bottomed dish (Matsunami Glass, Tokyo, Japan) and incubated at 37°C in a 5% CO₂ incubator overnight in FCS-DMEM. For study of endocytic kinetics, C2IIa was incubated with cells at 4°C for 1 h in FCS-DMEM. After three washes in cold FCS-DMEM, cells were transferred to FCS-DMEM containing C2I prewarmed to 37°C and incubated at the same temperature for various periods. They were washed four times with cold phosphate-buffered saline (PBS) and fixed with 4% paraformaldehyde at room temperature. For antibody labeling, the dishes were then incubated at room temperature for 15 min in 50 mM NH₄Cl in PBS and in PBS containing 0.1% Triton X-100 at room temperature for 20 min. After being washed with PBS containing 0.02% Triton X-100, the dishes were incubated at room temperature for 1 h with PBS containing 4% BSA, followed by rabbit anti-C2II antibody in PBS containing 4% BSA at room temperature for 1 h. After a wash with PBS containing 0.02% Triton X-100, the dishes were incubated at room temperature for 1 h with PBS containing 4% BSA and Alexa Fluor 488-conjugated anti-rabbit IgG. After being washed extensively with PBS containing 0.02% Triton X-100, the dishes were analyzed on a Leica TCS4D laser scanning confocal microscope. All images represent a single section through the focal plane. Akt was detected with rabbit anti-Akt antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG. Actin filaments were stained with rhodamine-phalloidin.

PI3K assay. The measurement of PI3K activity was performed as described previously (5, 9). MDCK cells treated with C2I plus C2IIa at 37°C for various periods were incubated with cell lysis buffer (137 mM NaCl, 20 mM Tris-HCl buffer [pH 7.4], 1 mM CaCl₂, 1 mM MgCl₂, 1% NP-40, and 1 mM sodium orthovanadate) for 20 min on ice. After the insoluble material was removed by centrifugation, the cell lysate (1 mg of protein) was incubated with an antibody against the p85 subunit of PI3K with 50 µl of protein G-Sepharose at 4°C for 2 h. The beads were washed twice with lysis buffer and twice with PI3K reaction buffer (25 mM Tris-HCl buffer [pH 7.4], 0.5 mM EGTA, and 100 mM NaCl). The washed beads were resuspended in 2.5 µl of PI3K reaction buffer with 0.5 µl of phosphatidylinositol (PI) dissolved in chloroform (20 mg/ml) to make micelles of PI. Assays were started with the addition of 2.5 µl of reaction start buffer (200 µM ATP and 200 mM MgCl₂) and 0.2 µl of [³²P]ATP (1 µCi/ml) and run at room temperature for 15 min. The reaction was stopped after the addition of 100 µl of a mixture of chloroform, methanol, and 11.6 N HCl (100:200:2). After centrifugation, the lower, organic phase was taken for thin-layer chromatography on silica gel plates (Merck), with development in chloroform, methanol, 25% ammonium hydroxide, and water (43:38:5:7). The plates were exposed to imaging plates and analyzed with BAS2000 film (Fuji Film). Quantification was done using Image Gauch (Fuji Film). Three individual MDCK cell combinations were analyzed.

Surface plasmon resonance (SPR) analysis. All experiments were performed with Biacore 3000 system sensor chips and their software evaluation package

FIG. 1. Sucrose density gradient analysis of ^{125}I -C2II-bound MDCK cells. MDCK cells were incubated with ^{125}I -C2IIa (500 ng/ml) in DMEM containing 10% FCS at 4°C for 1 h (A), washed, and incubated at 37°C for 30 min (B). MDCK cells were incubated with ^{125}I -C2II at 37°C for 30 min (C). The cells were extracted with HBSS containing 1% Triton X-100 at 4°C for 30 min, and sonicated. The extracts were mixed with 40% sucrose and then loaded at the bottom of a centrifuge tube. After sucrose gradient ultracentrifugation, 0.4-ml gradient fractions were collected from the tops of the tubes. Aliquots of the gradient fractions were dissolved in 2× SDS sample buffer and incubated at 37°C for 10 min. Samples were subjected to SDS-PAGE followed by autoradiography. (D) The distribution of cholesterol in the sucrose gradient fractions was determined as described in Materials and Methods. The data are means and standard deviations for four experiments. The aliquots of gradient fractions were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. (E) After the transfer, the blots were treated with anti-Lyn and anti-caveolin-1 antibodies. Peroxidase-conjugated secondary antibodies bound to the membrane were detected by enhanced chemiluminescence as described in Materials and Methods.

(Biacore KK, Tokyo, Japan) (23). The C2IIa oligomer was purified from trypsin-treated C2II by Mono Q chromatography with the same fast-performance liquid chromatography system as that used for Ib (23). Oligomers purified by this method are in a heptameric state, although the presence of monomers or lower-order oligomers has not been excluded. For CM5 chips, the system was maintained with a constant flow (10 $\mu\text{l}/\text{min}$) of HBS buffer (10 mM HEPES, pH 7.4, and 150 mM NaCl) at 25°C. C2IIa oligomers and C2IIa monomers were covalently bound to the carboxylated dextran matrix by amine coupling according to the manufacturer's directions (Biacore), except that they were diluted in sodium acetate buffer, pH 4.5, to a concentration of 500 nM. These dilutions were injected onto the activated surface at a flow rate of 2 $\mu\text{l}/\text{min}$ until the desired baseline level was reached (about 2,000 relative units) and then blocked with ethanolamine according to the manufacturer's directions. The interaction between immobilized C2IIa and C2I was examined at 25°C. C2I diluted in HBS was injected over the C2IIa oligomer's surface at 10 $\mu\text{l}/\text{min}$ for 170 s, allowing association to take place. Dissociation was then monitored in a constant flow of HBS for at least 150 s. Bound analyte was removed, and the C2IIa oligomer baseline was regenerated with a 10- μl pulse of 0.2 M glycine-HCl buffer (pH 2.0). The baseline decay was <3% per cycle. The association and dissociation rate constants k_{on} and k_{off} were determined from sensorgram data by using the BiaEvaluation 3.0 software package.

RESULTS

Binding of C2IIa to lipid rafts. To investigate binding of C2 toxin to lipid rafts of MDCK cells, ^{125}I -C2IIa was incubated

with MDCK cells in DMEM containing 10% fetal bovine serum at 4°C for 60 min and the cells were treated with 1% Triton X-100 at 4°C for 60 min. The membranes treated with Triton X-100 were fractionated by sucrose density gradient centrifugation. The fractions were subjected to SDS-PAGE and autoradiography. The C2IIa monomer (60 kDa) was found in the soluble fractions (fractions 6 to 10) more than in the insoluble fractions (fractions 3 to 5), and little oligomer of the toxin was detected, but what was formed was present in the soluble fractions (Fig. 1A). When MDCK cells preincubated with ^{125}I -C2IIa at 4°C for 60 min were incubated at 37°C for 30 min, the insoluble fractions were found to contain a band of high molecular mass, as expected for the C2IIa oligomer (Fig. 1B). On the other hand, when ^{125}I -C2II was incubated with the cells at 37°C for 30 min, the ^{125}I -C2II monomer was detected in the soluble fractions (Fig. 1C). To determine the molecular mass of the C2IIa oligomer, the sample containing C2IIa oligomer was dissolved in SDS sample buffer and analyzed by SDS-3.5% PAGE as described in Materials and Methods (data not shown). A molecular mass of 420 kDa was determined for the C2IIa oligomer. A similar result was reported by Barth et al. (7). The C2IIa oligomer mass of 420 kDa exceeds the

FIG. 2. Formation of oligomers of ¹²⁵I-labeled C2IIa in lipid rafts of MDCK cells. (A) MDCK cells were incubated with ¹²⁵I-labeled C2IIa at 37°C for the periods indicated. (B) MDCK cells were incubated with ¹²⁵I-labeled C2IIa plus C2I at 37°C for the periods indicated. Triton X-100-insoluble cell extracts were subjected to SDS-PAGE followed by autoradiography, as described in Materials and Methods. (C) The radioactivity of the ¹²⁵I-C2IIa oligomer in lipid rafts was determined for cells that were or were not treated with C2I, using a Fuji BAS2000 system. The data are means and standard deviations for three experiments.

monomer molecular mass (59.8 kDa) by about seven times, indicating that the oligomers should contain a C2IIa heptamer. Caveolin-1 and Lyn were detected in the insoluble fractions (fractions 3 to 5) (Fig. 1E), where >85% of the cholesterol was detected (Fig. 1D), showing that fractions 3 to 5 are lipid rafts. The result suggests that C2IIa forms an oligomer in the lipid rafts of the plasma membranes of cells at 37°C after binding of the monomer to membranes.

To determine the time course of the oligomer's formation in the lipid raft fractions, MDCK cells were incubated with ¹²⁵I-C2IIa at 37°C for various periods. As shown in Fig. 2A and C, the oligomer of C2IIa reached a maximum level after about 30 min of incubation and remained at a high level after 240 min.

To investigate the effect of C2I on the binding of C2IIa to lipid rafts, MDCK cells were incubated with the labeled C2IIa plus C2I at 37°C for various periods. As shown in Fig. 2B and C, the oligomer reached a maximum intensity after 30 min of incubation, later decreased in a time-dependent manner, and disappeared at 120 min. On the other hand, the treatment with C2I plus C2IIa or C2I alone did not change the distribution of Lyn and caveolin-1 in lipid rafts (data not shown). It therefore appears that the C2IIa oligomer disappears from lipid rafts in the presence of C2I, in a time-dependent manner.

Effect of MβCD on binding and biological activity of C2IIa. Kilsdonk et al. (17) reported that MβCD at concentrations of 5 to 10 mM selectively encapsulates cholesterol in membranes and does not deplete lipids other than cholesterol (23). The effect of MβCD on the cell rounding induced by C2 toxin was investigated (Table 1). When MDCK cells were incubated with 5 and 10 mM MβCD at 37°C for 60 min, the cholesterol content of the cells decreased to about 55 and 30%, respectively, of that in untreated cells (Table 1). Incubation of the toxin with MDCK cells pretreated with 5 and 10 mM MβCD at 37°C for 60 min resulted in reductions of about 50 and 90%, respectively, of the cell rounding of untreated cells induced by C2I plus C2IIa (Table 1). Next, we investigated whether C2IIa binds to cells treated with MβCD. The cells treated with 10 mM MβCD were incubated with ¹²⁵I-C2IIa at 37°C for 30 min. Figure 3A shows that little oligomer was detected in lipid raft fractions and that the monomer was found in non-lipid-raft fractions. As shown in Fig. 3B, the binding of C2IIa oligomer to lipid rafts was significantly decreased by the treatment with MβCD, in a concentration-dependent manner. However, cholesterol (50 μg/ml) had no effect on the rounding of untreated MDCK cells induced by C2 toxin (data not shown). Furthermore, when the ¹²⁵I-C2IIa was preincubated with cholesterol, the binding of labeled C2IIa to the cells was not blocked (data not shown), suggesting that C2IIa does not directly interact with cholesterol.

Binding of C2I to lipid rafts. We investigated the role of C2IIa in the binding of C2I to cells. MDCK cells were kept in the presence or absence of C2IIa at 4°C for 1 h, incubated with ¹²⁵I-C2I at 37°C for various periods, and then treated with 1% Triton X-100 at 4°C for 60 min. After sucrose density gradient centrifugation, the fractions were subjected to SDS-PAGE (Fig. 4A). One labeled band of about 49 kDa, which is the expected size of C2I, was detected in lipid rafts (fractions 3 to

TABLE 1. Effect of MbCD on cell rounding induced by C2I plus C2IIa^a

MbCD concn (mM)	Dose of C2IIa (ng/ml)				Amt (%) of cholesterol in cells ^b
	50	100	500	1,000	
0	+	++	+++	+++	100
5	—	+	++	+++	55.8 ± 8.1
10	—	—	+	++	31.3 ± 4.9

^a MDCK cells were treated with MbCD at 37°C for 30 min. The cells were incubated with various amounts of C2IIa in the presence of C2I (100 ng/ml). Cell rounding activity was scored as follows: +++, 100% rounding; ++, 50 to 80% rounding; +, 20 to 40% rounding; and —, no rounding. The experiments were repeated three times, and data from a representative experiment are shown.

^b The amount of cholesterol in cells was determined as described in Materials and Methods. The data are means and standard deviations for three experiments.

FIG. 3. Effect of M β CD on interaction of C2IIa with MDCK cells. (A) MDCK cells were incubated in the absence (control) or presence (10 mM M β CD) of 10 mM M β CD at 37°C for 30 min. ¹²⁵I-C2IIa was mixed with the cells and then incubated at 37°C for 60 min. Triton X-100-insoluble cell extracts were separated by sucrose density ultracentrifugation. Gradient fractions were subjected to SDS-PAGE followed by autoradiography, as described in Materials and Methods. (B) MDCK cells were preincubated in the absence and presence of 5 or 10 mM M β CD and then incubated with ¹²⁵I-C2IIa at 37°C for 60 min. The radioactivity of the ¹²⁵I-C2IIa oligomer in lipid rafts was determined using a Fuji BAS 2000 system. The data are means and standard deviations for four experiments. *, $P < 0.05$ compared with control; **, $P < 0.01$.

5) in the presence but not the absence of C2IIa, indicating that C2I binds to lipid raft fractions in the presence of C2IIa. Furthermore, the time course of ¹²⁵I-C2I's binding to the lipid rafts in the presence of C2IIa showed the same pattern as that of the ¹²⁵I-C2IIa oligomer's binding to lipid rafts in the presence of C2I (Fig. 2B and 4B). On the other hand, incubation of MDCK cells with ¹²⁵I-C2I in the presence of 50 mol of excess C2I at 37°C for 60 min resulted in little effect on the binding of labeled C2I to cells (data not shown), suggesting that the binding of C2I to the cells without C2IIa is nonspecific.

SPR analysis. We examined whether monomeric and oligomeric C2IIa molecules specifically interact with C2I. The SPR analysis showed that C2I bound to the oligomer of C2IIa in a dose-dependent manner (Fig. 5). The association rate constant (k_{on}) and the dissociation rate constant (k_{off}) for C2I were calculated to be $7.31 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ and $4.45 \times 10^{-3} \text{ M}^{-1} \text{ s}^{-1}$, respectively, and the equilibrium constant, K_d , calculated from these kinetic constants, was $6.27 \times 10^{-7} \text{ M}$ for C2IIa. However, as shown in Fig. 5, C2I did not bind to the monomer of C2IIa. Furthermore, C2I did not bind to a protein-free surface blocked with ethanolamine. This result suggests that C2I docks with the cell-bound C2IIa oligomer.

Internalization of C2IIa into MDCK cells. To investigate the internalization of C2IIa into MDCK cells, the cells were incubated with C2IIa (1 $\mu\text{g}/\text{ml}$) at 4°C for 60 min, washed, and incubated with C2I (1 $\mu\text{g}/\text{ml}$) at 37°C for specific periods. After incubation at 4°C for 60 min, as shown in Fig. 6, an immuno-

FIG. 4. Binding of ¹²⁵I-C2I to lipid rafts of MDCK cells in the presence of C2II. (A) MDCK cells were incubated in the absence or presence of C2IIa (500 ng/ml) at 4°C for 60 min, washed, and incubated with ¹²⁵I-labeled C2I (500 ng/ml) at 37°C for 60 min. Triton X-100-insoluble cell extracts were separated by sucrose density ultracentrifugation and analyzed as described in Materials and Methods. (B) The radioactivity of ¹²⁵I-C2I bound to lipid rafts was determined at 37°C for the periods indicated, using a Fuji BAS2000 system. The data are means and standard deviations for three experiments.

fluorescent signal for C2IIa was found in the plasma membranes, but no signal was detected in the cytosol (0 min). After incubation for 15 min at 37°C, the signal for C2IIa on the surface decreased and appeared as small intracellular vesicles located near the plasma membranes. After 30 min, C2IIa was no longer detected on membranes but was observed in intracellular vesicles. Furthermore, the disappearance of the cortical actin was concomitant with the internalization of C2IIa in the cells.

Activation of the PI3K-Akt pathway by C2 toxin. It has been reported that activation of the PI3K-Akt signaling pathway plays a role in the entry of a subset of intracellular pathogens

FIG. 5. SPR analysis of binding of C2I to C2IIa immobilized on a dextran matrix flow cell surface by primary amine coupling. Injections of C2I (1 to 10 μM) in HBSS were made onto the C2IIa oligomer surface. The binding of C2I was recorded in real time. Injections were done at 10 $\mu\text{l}/\text{min}$ for 170 s, followed by HBSS alone. The experiments were repeated three times, and results for a representative experiment are shown.

FIG. 6. Internalization of C2IIa in MDCK cells. MDCK cells were incubated with C2IIa (1 μ g/ml) at 4°C for 1 h, washed, and incubated with C2I (1 μ g/ml) at 37°C for the periods indicated. Cells were fixed, permeabilized, and stained with anti-C2II antibody and rhodamine-phalloidin. C2IIa and actin were viewed with a confocal microscope. The experiments were repeated three times, and representative results are shown.

(16, 26, 27). We investigated the involvement of PI3K in the endocytosis of C2 toxin. LY294002, an inhibitor of PI3K, inhibited C2IIa endocytosis, but LY303511 (negative control for LY294002) did not (Fig. 7). Wortmannin and quercetin (inhibitors of PI3K) also blocked the internalization of C2IIa (data not shown). These observations suggest that PI3K inhibitors block the endocytosis of C2IIa. We analyzed whether C2 toxin affects PI3K activity in MDCK cells. Incubation of the

FIG. 7. Effects of PI3K inhibitors on internalization of C2 toxin into MDCK cells. MDCK cells were treated with 10 μ M LY294002 or 10 μ M LY303511 at 37°C for 1 h and then rinsed. C2IIa (1 μ g/ml) was added at 4°C for 1 h. Cells were rinsed and incubated with C2I (1 μ g/ml) at 37°C for 0 and 30 min. Cells were fixed, permeabilized, and stained with anti-C2IIa antibody. C2IIa was viewed with a confocal microscope. The experiments were repeated three times, and representative results are shown.

FIG. 8. Effect of C2 toxin on PI3K activity in MDCK cells. (A) MDCK cells were treated with C2I (1 μ g/ml) alone (○), C2IIa (1 μ g/ml) alone (■), and C2I (1 μ g/ml) plus C2IIa (1 μ g/ml) (●) at 37°C for the periods indicated. (B) MDCK cells were incubated with 10 μ M LY294002 or 10 μ M LY303511 at 37°C for 1 h and treated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) at 37°C for 30 min. The cell lysates were subjected to immunoprecipitation with an antibody against p85 α of PI3K. PI3K activities in immunoprecipitates from C2 toxin-treated and control cells were determined as described in Materials and Methods. Data are means $\pm$ standard deviations for three independent experiments. *, $P < 0.05$ for comparison between dimethyl sulfoxide (DMSO) plus C2 toxin and LY294002 plus C2 toxin.

cells with C2I plus C2IIa at 37°C resulted in the activation of PI3K within 30 min, but that with C2IIa alone or C2I alone did not (Fig. 8A). The activation of PI3K by C2I plus C2IIa was attenuated by treatment with LY294002 but not by that with LY303511 (Fig. 8B). Furthermore, LY294002, wortmannin, and quercetin inhibited the cell rounding induced by C2I plus C2IIa, but LY303511 did not (data not shown). These results suggest that the internalization of C2 toxin is linked to the activation of PI3K.

Phosphorylation of PDK1 and Akt by C2 toxin. It is known that the activation of PI3K results in the phosphorylation of PDK1, which activates Akt (34). To test whether treatment of MDCK cells with toxin induces activation of PDK1 and Akt, the cells were incubated with C2I plus C2IIa, C2I alone, or C2IIa alone at 37°C for the indicated periods. The treated cells were subjected to SDS-PAGE, and the proteins were analyzed by Western blotting with anti-phospho-PDK1

FIG. 9. Phosphorylation of PDK1 induced by C2 toxin in MDCK cells. MDCK cells were incubated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) (A), with C2I alone (1 μ g/ml) (B), or with C2IIa (1 μ g/ml) alone (C) at 37°C for the periods indicated. (D) MDCK cells treated with 10 μ M LY294002 or 10 μ M LY303511 at 37°C for 1 h were incubated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) at 37°C for the periods indicated. The extracted phospho-PDK1 and PDK1 were subjected to SDS-PAGE and Western blotting using specific antibodies. Typical results from one of four experiments are shown.

(Fig. 9) and anti-phospho-Akt (Fig. 10) antibodies. Phosphorylation of PDK1 (Fig. 9A) and Akt (Fig. 10A) reached a maximum within 5 and 15 min, respectively, under the conditions used. This result indicated that incubation of the cells with C2I plus C2IIa resulted in phosphorylation of PDK1 and, later, Akt. However, when the cells were incubated with C2IIa alone or C2I alone, PDK1 and Akt were not phosphorylated (Fig. 9B and 10B). The increase in phosphorylated protein was not because of an increase in the protein concentration of PDK1 or total Akt protein, as evident from the similar intensities of bands when blots were probed with an antibody that recognized both the phosphorylated and nonphosphorylated forms.

Next, we examined the effects of inhibitors of PI3K and Akt on the phosphorylation of these proteins induced by C2I plus C2IIa. LY294002 inhibited the phosphorylation of PDK1 and Akt induced by C2I plus C2IIa, but LY303511 did not (Fig. 9D). Furthermore, LY294002 and Akt inhibitor X inhibited the phosphorylation of Akt induced by C2I plus C2IIa (Fig. 10D). These results suggested that the phosphorylation of Akt induced by C2 toxin was dependent upon the activation of PI3K. It has been reported that after the activation of PI3K, Akt translocates to the membranes, where it is phosphorylated by PDK1. Phosphorylated Akt moved from the membrane, through the cytosol, and to the nucleus (34). To determine whether Akt is recruited to the membrane during the endocy-

FIG. 10. Phosphorylation of Akt induced by C2 toxin in MDCK cells. MDCK cells were incubated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) (A), with C2I alone (1 μ g/ml) (B), or with C2IIa (1 μ g/ml) alone (C) at 37°C for the periods indicated. (D) MDCK cells treated with DMSO, 10 μ M LY294002, 10 μ M LY303511, or 10 μ M Akt inhibitor X at 37°C for 1 h were incubated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) at 37°C for the periods indicated. The extracted phospho-Akt and Akt were subjected to SDS-PAGE and Western blotting using specific antibodies. Typical results from one of four experiments are shown.

tosis of C2 toxin, we incubated MDCK cells with C2I plus C2IIa at 37°C for the periods indicated in Fig. 11. Akt was localized to the nucleus without C2 toxin. Following incubation with C2I plus C2IIa for 15 min, Akt was recruited to the plasma membranes. C2IIa alone did not induce the recruitment of Akt (data not shown). After incubation with C2I plus C2IIa for 30 min, Akt started to be relocated to the nucleus. Akt inhibitor X blocked the recruitment of Akt induced by C2I plus C2IIa, as shown in Fig. 11.

To investigate the effects of Akt inhibitor X on the endocytosis of C2 toxin, MDCK cells were pretreated with Akt inhibitor X at 37°C for 2 h. Figure 12 shows the internalization of C2IIa in the absence and presence of Akt inhibitor X. MDCK cells preincubated with C2I plus C2IIa without Akt inhibitor X exhibited a loss of cortical actin (F-actin). However, treatment of the cells with C2I plus C2IIa in the presence of the inhibitor did not result in the disappearance of F-actin. Akt inhibitor X

FIG. 11. Reversible translocation of Akt by C2 toxin in MDCK cells. (A) MDCK cells were incubated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) at 37°C for the periods indicated. (B) MDCK cells were pretreated with 10 μ M Akt inhibitor X at 37°C for 1 h. The cells were then incubated with C2I (1 μ g/ml) and C2IIa (1 μ g/ml) at 37°C for 15 min. Cells were fixed, permeabilized, and stained with anti-Akt antibody. Akt was viewed with a confocal microscope. The experiments were repeated three times, and representative results are shown.

inhibited the cell-rounding activity induced by the C2 toxin of the control (data not shown) but did not inhibit C2 toxin endocytosis.

DISCUSSION

The present study demonstrates that (i) C2IIa binds to whole membranes of MDCK cells and then later accumulates on lipid rafts and forms oligomers, (ii) C2I binds to the oligomers of C2IIa on the rafts, and then (iii) the activation of PI3K and Akt by the C2I-C2IIa complex is necessary for endocytosis of the complex into cells.

The monomer of C2IIa was detected in the Triton X-100-soluble and -insoluble fractions of MDCK cells incubated with C2IIa at 4°C, suggesting that the receptor of C2IIa is distributed around cytoplasmic membranes. Thus, it is unlikely that the receptor is specifically localized to lipid rafts. After incubation at 37°C, the oligomer of C2IIa was detected on lipid rafts, suggesting that the monomer bound to the receptor is accumulated at lipid rafts and that the oligomer is formed on lipid rafts. It therefore appears that a receptor which is linked with C2IIa gathers in lipid rafts at 37°C and that C2IIa forms oligomers on the lipid rafts.

Treatment of MDCK cells with M β CD reduced the cholesterol content in lipid raft fractions, the binding of C2IIa to the cells, and the rounding activity induced by C2I plus C2IIa. However, cholesterol had no effect on the activity induced by C2I plus C2IIa, showing that C2IIa does not interact directly with cholesterol in lipid rafts. It has been speculated that the functional properties of lipid rafts that are relevant to the binding and intracellular trafficking of various toxins may be especially susceptible to treatment with M β CD (1, 20, 22, 24, 35). It has been reported that the disruption or depletion of cell membrane-associated cholesterol causes major changes in the function and/or distribution of raft-associated membrane components (20, 30). It therefore appears that the inhibition of the event induced by C2I plus C2IIa by M β CD could be due to changes in the properties of lipid rafts that occur when cholesterol is removed by M β CD, suggesting that a function of lipid rafts is to gather C2IIa so that it forms oligomers.

In the present study, C2I was detected mainly with C2IIa oligomers in the raft fractions. Furthermore, SPR analysis showed that C2I binds to the C2IIa oligomer, but not the monomer. Thus, it appears that C2I binds specifically to the oligomers of C2IIa on the rafts of the cells. We reported that Ia binds to the oligomer, but not the monomer, of Ib (23). Milne et al. (19) also reported that *B. anthracis* lethal factor binds to the oligomer of PA, but not the monomer. These observations show that the binding of C2I to C2IIa oligomers formed on lipid rafts is the same as that of the enzymatic component to oligomers of the binding component, such as in the case of iota-toxin and *B. anthracis* toxin.

Several bacterial pore-forming toxins have been reported to utilize lipid rafts to intoxicate cells. PA (2) and Ib (23) are reported to associate with the receptors on nonlipid rafts and to form functional oligomers on lipid rafts. Aerolysin (1) and *Clostridium septicum* alpha-toxin (13) are known to bind to

FIG. 12. Effects of Akt inhibitors on internalization and cytotoxicity of C2IIa in MDCK cells. MDCK cells were treated with DMSO or 10 μ M Akt inhibitor X at 37°C for 1 h and then rinsed. C2IIa (1 μ g/ml) was added at 4°C for 1 h. Cells were rinsed and incubated with C2I (1 μ g/ml) at 37°C for 30 min. Cells were fixed, permeabilized, and stained with anti-C2IIa antibody and rhodamine-phalloidin. C2IIa was viewed with a confocal microscope. The experiments were repeated three times, and representative results are shown.

glycosylphosphatidylinositol-anchored proteins in lipid rafts, and *C. perfringens* epsilon-toxin (20) and perfringolysin (35) oligomerize in cholesterol-containing lipid rafts. It has been proposed that lipid rafts serve as concentrating platforms to promote the formation of pores by toxins that form oligomers. The present study indicates that the internalization of C2 toxin is mediated through lipid rafts (cholesterol-rich microdomains) at the plasma membranes, suggesting that the lipid rafts contain all of the necessary components for the mediation of endocytosis. Accordingly, the C2I-C2IIa complex seems to be internalized in the cells by endocytosis.

CIIa was detected on the cell surface at 4°C and, after 15 min at 37°C, in vesicles in the cytosol. Coinciding with the internalization of C2IIa, a loss of F-actin was observed. Barth et al. (7) reported that the cytosolic delivery of C2I is blocked by bafilomycin. Haug et al. (15) reported that studies of C2 toxin in intracellular compartments revealed a colocalization with the early endosome marker Rab5. From these observations, it appears that after its internalization, the C2I-C2IIa complex is trafficked to the early endosomes, and that C2I is released from the vesicles to the cytosol.

Several workers have reported that the PI3K-Akt signaling pathway is involved in diverse processes, such as vesicular trafficking, mitogenesis, cell survival, and microbial entry (10). PI3K catalyzes the production of phosphatidylinositol 3,4,5-triphosphate [PtdIns(3,4,5)P₃] at cellular membranes and contributes to the recruitment and activation of different intracellular signaling components, such as PDK1 and Akt, bearing a pleckstrin homology domain, in response to a variety of stimuli (34). LY294002 blocked the activation of PI3K induced by the toxin. The inhibition by LY294002 also blocked the internalization of C2IIa. Therefore, it is likely that C2I plus C2IIa activates PI3K before endocytosis. These results suggested that the activation of PI3K is required for endocytosis and that subsequent production of PtdIns(3,4,5)P₃ is necessary for internalization of the toxin. Both PI3K and Akt have been shown to modify cytoskeletal dynamics, to be involved in the regulation of membrane traffic (10), and to have direct roles in macropinocytosis, a pathway used by some pathogenic bacteria for cellular entry (25, 37). PDK1 and Akt are recruited to the inner leaflet of the plasma membrane through the binding of the pleckstrin homology domain to PtdIns(3,4,5)P₃, which is a product of PI3K (34). Since both PDK1 and Akt interact with PtdIns(3,4,5)P₃, PDK1 colocalizes with Akt (34). The interaction between PDK1 and the C terminus of Akt leads to autophosphorylation and activation of PDK1 (31). Following its activation, Akt is phosphorylated at serine 473 and at threonine 308 by PDK1, leading to activation of Akt (34). The activated Akt is translocated from the plasma membranes through the cytosol to the nucleus (34). In the present study, C2I plus C2IIa led to the phosphorylation of PDK1 and, later, Akt. Our findings indicate that docking of C2I onto the membrane-bound C2IIa oligomer induces the initiation of endocytosis. Phosphorylation of PDK1 and Akt induced by the toxin was inhibited by LY294002, and that of Akt was inhibited by Akt inhibitor X. C2I plus C2IIa induced the translocation of Akt from the cytosol to the plasma membranes and the relocalization of activated Akt to the nucleus. Akt inhibitor X blocked the release of C2I from the early endosomes into the cytosol and the translocation of Akt to membranes induced by

C2I plus C2IIa. It therefore appears that the toxin-induced activation of Akt is involved in the release of C2I from the endosomes to the cytosol. Inhibitors of PI3K, i.e., LY294002, wortmannin, and quercetin, and Akt inhibitor X also inhibited the cytotoxicity of C2 toxin, supporting the observation that cytotoxicity is mediated via activation of the PI3K-Akt signaling pathway in MDCK cells.

In conclusion, the binding of C2I to the oligomer of C2IIa on lipid rafts triggers the activation of PI3K and, in turn, initiation of endocytosis. The subsequent phosphorylation of PDK1 and Akt is implicated in the translocation of C2I from the early endosomes into the cytosol.

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