Binding and internalization of Clostridium botulinum C2 toxin.

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**Abstract**

*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 a receptor-mediated endocytosis. We investigated the binding to and internalization of C2IIa in cells. The C2IIa monomer formed oligomers on lipid rafts in membranes of MDCK cells. Methyl-beta-cyclodextrin (MbCD) 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 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 into the cells and the rounding activity of C2I plus C2IIa. Incubation of the cells with C2I plus C2IIa resulted in the activation of PI3K, and phosphorylation of phosphoinositide-dependent kinase 1 (PDK1) 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 internalization of C2IIa. The results suggest that the binding of C2I to the oligomer of C2IIa on the rafts triggers the activation of the PI3K/Akt signaling pathway and in turn initiation of endocytosis.
Introduction

*Clostridium botulinum* produces botulinum C2 toxin that recruits 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 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 at arginine-177 in the cytosol (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 that 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 interaction with the enzymatic component (lethal factor or edema factor), domain 2 in the 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 surface 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 its receptor via a lipid raft-mediated process (2, 23). However, little is known
about the binding to and initial entry into the cells of 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 the 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-PDK1 and anti-PDK1 antibodies were purchased from Cell Signaling (Danvers, MA). Mouse anti-caveolin-1 and Lyn antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-

PI3K p85 antibody was purchased from Upstate Biotechnology (Charlottesville, VA). Horseradish peroxidase-labeled anti-rabbit IgG, horseradish peroxidase-labeled anti-mouse IgG, the pGEX4T-1 vector, Protein G-Sepharose and the enhanced chemiluminescence (ECL) kit were from GE Healthcare (Tokyo, Japan). 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 for C2I,
the primers C2I-01 (5'-GGAGATCTATGCCAATAAATAAGAA-3'), containing a Bgl II site (in bold), and C2I-02 (5'-CCGTCGACCTAAATCTCTTTATT-3'), containing a Sal I site (in bold), and for C2II, the primers C2II-01 (5'-GCTTCGGGATCCATGTAGTTTCTAAAATTGAG-3'), containing a Bam HI site (in bold), and C2I-02 (5'-TCGATCCTCGAGCTATATTATTTATCTAA-3'), containing a Xho I site (in bold) (12, 18). The PCR product was 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 Bgl II/Sal I and Bam HI/Xho I, respectively, and cloned into Bam HI/Sal I-digested- and Bam HI/Xho I-digested-pGEX4T-1, resulting in pGEX4T-C2I and pGEX4T-C2II. Recombinant C. botulinum toxin 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 or -C2II antiserum. The antisera of 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 control (ethanol alone at the corresponding dilution) at 37 °C for 30 min to use in the assay. Final ethanol concentration is 0.1%. To measure the effect 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/mm; 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 the 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 xg) at 4 °C for 18 h in a SW55 rotor (Beckman Instruments, Inc., Palo Alto, CA), and fractionated from the top (0.4 ml/fraction). The aliquots
were subjected to 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 Na,VO₄ and

kept on ice for 30 min. The precipitate was collected by centrifugation at 10,000 x g for 20

min and heated in 2 % SDS-sample buffer at 99 °C for 3 min. The samples were

electrophoresed on a SDS-PAGE gel, and transferred to a PVDF 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 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 MbCD in HBSS, 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 C2Ila oligomer by SDS-3.5% PAGE. To determine the molecular weight of C2Ila oligomer, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in a 3.5% gel was performed according to the method as described previously (21). High-molecular-mass standards for SDS-PAGE were phosphorylase b cross-linked SDS molecular weight markers (Sigma): monomer (97 kDa), dimer (194 kDa), trimer (292 kDa), tetramer (390 kDa), pentamer (487 kDa), and hexamer (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-bottom dish (Matsunami Glass, Tokyo, Japan) at 37 °C in a 5% CO₂ incubator overnight in FCS/DMEM. For studying endocytic kinetics, C2Ila 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 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% bovine serum albumin (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 extensive washing 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 (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 γ³²P-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 of HCl (100:200:2). After centrifugation, the lower organic phase was taken for thin layer chromatography (TLC) on Silica Gel plates (Merck) to be developed in chloroform, methanol, 25% ammonium hydroxide and water (43:38:5:7). The plates were exposed to imaging plates and analyzed by BAS2000 (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 (Biacore KK, Tokyo, Japan) (23). The C2IIa oligomer was purified from trypsin-treated C2II by Mono Q chromatography with the fast-performance liquid chromatography system as 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 µl/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 μl/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 μl/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 (no.6 to 10) more than the insoluble fractions (no.3 to 5) and little oligomer of the toxin was detected but what was formed was present in the soluble fractions (Figure 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 weight, as expected for the C2IIa oligomer (Fig. 1B). To determine the molecular mass of C2IIa oligomer, the sample containing C2IIa oligomer was dissolved in SDS sample buffer and analyzed by SDS-3.5 % PAGE as described in Methods and Materials (data not shown). A molecular mass for C2IIa oligomer of 420 kDa was determined. A similar result was reported by Barth et al. (7). The C2IIa oligomer mass of 420 kDa exceeds the monomer molecular mass (59.8 kDa) about 7 times, indicating that the oligomers should contain a C2IIa heptamer. Caveolin-1 and Lyn were detected in the
insoluble fractions (no. 3 to 5) (Fig. 1D) where >85 % of the cholesterol was detected (Fig.
1C), showing that the fractions 3-5 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 the 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 ^125^I-C2IIa at 37 °C for various periods. As shown in Fig. 2A
and 2C, 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 2C, 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 MbCD on binding and biological activity of C2IIa.** Kilsdonk et al. (17) reported
that MbCD selectively encapsulates cholesterol in membranes and does not deplete lipids
other than cholesterol at concentrations of 5 to 10 mM (23). The effect of MbCD on the cell
rounding induced by C2 toxin was investigated (Table 1). When MDCK cells were incubated
with 5 or 10 mM MbCD 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 or 10 mM MbCD at 37 °C for 60 min resulted in a
reduction 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
MbCD. The cells treated with 10 mM MbCD were incubated with 125I-C2IIa at 37 °C for 30
min. Figure 3(A) shows that little oligomer was detected in lipid raft fractions and the
monomer was found in non-lipid raft fractions. As shown in Fig. 3(B), the binding of C2IIa
oligomer to lipid rafts was significantly decreased by the treatment with MbCD 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, then
incubated with 125I-C2I at 37 °C for various periods and 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 (no. 3 to 5) in the presence, but not absence of C2IIa, indicating that C2I
binds to lipid raft fractions in the presence of C2IIa. Furthermore, the time course of $^{125}$I-C2I’s
binding to the lipid rafts in the presence of C2IIa showed the same pattern as that of the $^{125}$I-
C2IIa oligomer’s binding to lipid rafts in the presence of C2I (Fig. 2B and Fig. 4B). On the
other hand, incubation of MDCK cells with $^{125}$I-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 non-specific.

**SPR analysis.** We examined whether monomeric and oligomeric C2IIa 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 $7.31 \times 10^3$ M$^{-1}$ s$^{-1}$ and $4.45 \times 10^{-3}$ s$^{-1}$, respectively,
and the equilibrium constant, $K_{d}$, calculated from these kinetic constants, was $6.27 \times 10^{-7}$ 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. The result
suggests that C2I docks with the cell-bound C2IIa oligomer.
Internalization of C2IIa into MDCK cells. To investigate the internalization of C2IIa in MDCK cells, the cells were incubated with C2IIa (1 µg/ml) at 4 °C for 60 min, washed, and incubated with C2I (1 µg/ml) at 37 °C for specific periods. After the incubation at 4 °C for 60 min as shown in Fig. 6, an immunofluorescent 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 (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 of LY294002) did not (Fig. 7). Wortmannin and quercetin (inhibitors of PI3K) also blocked the internalization of C2IIa (data not shown). The observation suggests that PI3K inhibitors block the endocytosis of C2IIa. We analyzed whether C2 toxin affects PI3K activity in MDCK cells. Incubation of the cells with C2I plus C2IIa at 37 °C resulted in the activation of
PI3K within 30 min, but that with C2Ia alone or C2I alone did not (Fig. 8A). The activation
of PI3K by C2I plus C2IIa was attenuated by treatment with LY294002, but not 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 the 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) and anti-phospho Akt (Fig. 10) antibodies. Phosphorylation of PDK1
(Fig. 9A) and Akt (Fig. 10A) reached a maximum within 5 and 15 min under the conditions,
respectively. The 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 Fig. 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 intensity of bands when blots were
probed with the antibody that recognized both the phosphorylated and nonphosphorylated forms.

Next, we examined the effect 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. 9C). Furthermore, LY294002 and Akt inhibitor X inhibited the phosphorylation of Akt induced by C2I plus C2IIa (Fig. 10C). The result 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 to the nucleus through the cytosol (34). To determine whether Akt is recruited to the membrane during the endocytosis 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 the 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 relocalized 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 inhibited the cell-rounding activity of 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 the 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 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 C2IIa forms oligomers in the lipid rafts.

Treatment of MDCK cells with MβCD 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 directly interact with cholesterol in lipid rafts. It has been speculated that the functional properties of lipid rafts that are relevant to the binding and the intracellular
trafficking of various toxins may be especially susceptible to treatment with MβCD (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 MβCD could be due to changes in the properties of lipid rafts that occur when cholesterol is removed by MβCD, suggesting that a function of lipid rafts is to gather C2IIa so that it forms oligomers.

In the present study, C2I was mainly detected 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 monomer of Ib (23). Milne et al. (19) also reported that \textit{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 the lipid rafts is the same as that of the enzymatic component to oligomers of the binding component, such as iota-toxin and \textit{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 non-lipid rafts and
form functional oligomers on lipid-rafts. Aerolysin (1) and *Clostridium septicum* alpha-toxin (13) are known to bind to 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, 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 production of 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 (PH) 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
the endocytosis, and 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 its PH domain to PtdIns(3,4,5)P₃ which is a product of PI3K (34). As both PDK1 and Akt
interact with PtdIns(3,4,5)P₃, PDK1 co-localizes 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 to the nucleus through the cytosol (34). In the present study, C2I plus C2IIa led to
the phosphorylation of PDK1 and later, that of 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 to 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
cytosol. Inhibitors of PI3K, LY294002, wortmannin and quercetin, and Akt inhibitor X also
inhibited the cytotoxicity of C2 toxin, supporting that the 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 the 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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Figure legends

Figure 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% fetal calf serum at 4 °C for 1 h (A), washed, and incubated at 37 °C for 30 min (B), then 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 x SDS sample buffer and incubated at 37 °C for 10 min. Samples were subjected to SDS-PAGE, followed by autoradiography. The distribution of cholesterol in the sucrose gradient fractions was determined as described in Materials and Methods (C). The data are the means and standard deviations from four experiments. The aliquots of gradient fractions were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. After the transfer, the blots were treated with anti-Lyn and anti-caveolin-1 antibodies (D). Peroxidase-conjugated secondary antibodies bound to the membrane were detected by enhanced chemiluminescence as described in Materials and Methods.
Figure 2. Formation of the oligomer of $^{125}$I-labeled C2IIa in lipid rafts of MDCK cells.

(A) MDCK cells were incubated with $^{125}$I-labeled C2IIa at 37 °C for the periods indicated.

(B) MDCK cells were incubated with $^{125}$I-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 $^{125}$I-C2IIa oligomer in lipid rafts was determined for cells that were or not treated with C2I using the Fuji BAS2000 system. The data are the means and standard deviations from three experiments.

Figure 3. Effect of MbCD on interaction of C2IIa with MDCK cells.

(A) MDCK cells were incubated in the absence (Control) or presence (10 mM MbCD) of 10 mM MbCD at 37 °C for 30 min. $^{125}$I-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 MbCD and then incubated with $^{125}$I-C2IIa at 37 °C for 60 min. The radioactivity of the $^{125}$I-C2IIa oligomer in lipid rafts was determined using the
Fuji BAS 2000 system. The data are the means and standard deviations from four experiments.

*P<0.05 and **P<0.01, compared with control.

Figure 4. Binding of ^125^I-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 ^125^I-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 ^125^I-C2I bound to lipid rafts was determined at 37 °C for the periods indicated using a Fuji BAS2000 system. The data are the means and standard deviations from three experiments.

Figure 5. SPR analysis of binding of C2I to C2IIa immobilized on a dextran matrix flow cell surface by primary amine coupling.

Injections of C2I (1-10 μM) in HBSS were made onto the C2IIa oligomer surface. The binding of C2I was recorded in real time. Injections were done at 10 μl/min for 170 s, followed by HBSS alone. The experiments were repeated three times, and results for a representative experiment are shown.
Figure 6. Internalization of C2IIa to MDCK cells.

MDCK cells were incubated with C2IIa (1 µg/ml) at 4 °C for 1 h, washed, and incubated with C2I (1 µg/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 a representative result is shown.

Figure 7. Effect of PI3K inhibitors on internalization of C2 toxin into MDCK cells.

MDCK cells were treated with 10 µM LY294002 or 10 µM LY303511 at 37 °C for 1 hr and then rinsed. C2IIa (1 µg/ml) was added at 4 °C for 1 h. Cells were rinsed and incubated with C2I (1 µg/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 a representative result is shown.

Figure 8. Effect of C2 toxin on PI3K activity in MDCK cells.

(A) MDCK cells were treated with C2I (1 µg/ml) alone (○), C2IIa (1 µg/ml) alone (■), and...
C2I (1 µg/ml) plus C2IIa (1 µg/ml) (●) at 37 °C for the periods indicated. (B) MDCK cells were incubated with 10 µM LY294002 or 10 µM LY303511 at 37 °C for 1 hr and treated with C2I (1 µg/ml) and C2IIa (1 µg/ml) at 37 °C for 30 min. The cell lysates were subjected to immunoprecipitation with the antibody against p85α of PI3K. PI3K activity in immunoprecipitates from C2 toxin-treated and control cells was determined as described in Materials and Methods. Data are the mean ± s.d. for three independent experiments. Comparison between DMSO plus C2 toxin and LY294002 plus C2 toxin: *P<0.05

Figure 9. Phosphorylation of PDK1 induced by C2 toxin in MDCK cells.

MDCK cells were incubated with C2I (1 µg/ml) and C2IIa (1 µg/ml) (A), C2I alone (1 µg/ml) (B) or C2IIa (1 µg/ml) alone (C) at 37 °C for the periods indicated. MDCK cells treated with 10 µM LY294002 or 10 µM LY303511 at 37 °C for 1 h were incubated with C2I (1 µg/ml) and C2IIa (1 µg/ml) at 37 °C for the periods indicated (D). The extracted phospho-PDK1 and PDK1 were subjected to SDS-PAGE and Western blotting using specific antibodies. A typical result from one of four experiments is shown.

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

Figure 11. Reversible translocation of Akt by C2 toxin in MDCK cells.

(A) MDCK cells were incubated with C2I (1 μg/ml) and C2IIa (1 μg/ml) at 37 °C for the periods indicated. (B) MDCK cells were pretreated with 10 μM Akt inhibitor X at 37 °C for 1 h. The cells were incubated with C2I (1 μg/ml) and C2IIa (1 μg/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 a representative result is shown.

Figure 12. Effect of Akt inhibitors on internalization and cytotoxicity of C2IIa on MDCK cells.

MDCK cells were treated with DMSO or 10 μM Akt inhibitor X at 37 °C for 1 h and then
rinsed. C2IIa (1 µg/ml) was added at 4 °C for 1 h. Cells were rinsed and incubated with C2I (1 µg/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 a representative result is shown.
Figure 1  Nagahama et al.
Figure 2 Nagahama et al.

(A) $^{125}$I-C2IIa

(B) $^{125}$I-C2IIa + C2I

(C) Binding of $^{125}$I-C2IIa oligomer in lipid rafts
Table 1 Effect of MbCD on cell rounding induced by C2I plus C2IIa

<table>
<thead>
<tr>
<th>MbCD (mM)</th>
<th>Dose of C2IIa (ng/ml)</th>
<th>% of cholesterol in cellsa</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>5</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>10</td>
<td>-</td>
<td>+</td>
</tr>
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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; –, no rounding. The experiments were repeated three times, and data from a representative experiment are shown.

aThe amount of cholesterol in cells was determined as described in Materials and Methods. The data are means and standard deviations for three experiments.
Figure 3  Nagahama et al.

(A) Control

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<tr>
<td>1</td>
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<tr>
<td>(kDa)</td>
<td>250</td>
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10 mM MbCD

<table>
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<th>Fraction number</th>
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<tbody>
<tr>
<td>1</td>
<td>2</td>
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<tr>
<td>(kDa)</td>
<td>250</td>
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(B) $^{125}$I-C2IIa oligomer in rafts (cpm)

![Graph showing $^{125}$I-C2IIa oligomer in rafts (cpm) for Control, 5 mM MbCD, and 10 mM MbCD](image)

- Control
- 5 mM MbCD
- 10 mM MbCD

* p < 0.05
** p < 0.01
Figure 4  Nagahama et al.

(A) Top Fraction number Bottom

125I- C2I

125I- C2I + C2IIa

(B) 125I-C2I binding in rafts (cpm)

0 60 120 180 240
Figure 5 Nagahama et al.

C2I (µM)

Reflectance units

Time (sec)

C2I (2 µM) on C2IIa monomer surface

C2I (2 µM) on surface lacking C2IIa
Figure 6  Nagahama et al.

37 °C  0 min  15 min  30 min

C2IIa

Actin

Overlay
Figure 7  Nagahama et al.

LY294002
LY303511
Figure 8  Nagahama et al.

(A) Graph showing PI 3-kinase activity over time (min) with different treatments including DMSO, LY294002, and LY303511.

(B) Bar graph comparing PI 3-kinase activity with treatments: None, DMSO, LY294002, LY303511, C2I plus C2IIa.
Figure 9  Nagahama et al.

(A) C2I plus C2IIa

(B) C2I

(C) C2IIa

(D) LY303511  LY294004

min  0 2 5 15 30 60

min  0 5 15 0 5 15
Figure 10 Nagahama et al.

(A) C2I plus C2IIa

(B) C2I

(C) C2IIa

(D) DMSO and Akt inhibitor

LY294002 and Akt inhibitor

LY30511 and Akt inhibitor
Figure 11 Nagahama et al.
Figure 12 Nagahama et al.

C2IIa

F-actin

Merge

None

Akt inhibitor X