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Research Article

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Adenosine Diphosphate–Ribosylation of G-Actin by Botulinum C2 Toxin Increases Endothelial Permeability In Vitro

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Abstract

The endothelial cytoskeleton is believed to play an important role in the regulation of endothelial permeability. We used botulinum C2 toxin to perturb cellular actin and determined its effect on the permeability of endothelial cell monolayers derived from porcine pulmonary arteries. The substrate for botulinum C2 toxin is nonmuscle monomeric actin which becomes ADP-ribosylated. This modified actin cannot participate in actin polymerization and, in addition, acts as a capping protein. Exposure of endothelial cell monolayers to botulinum C2 toxin resulted in a dose- (3–100 ng/ml) and time-dependent (30–120 min) increase in the hydraulic conductivity and decrease in the selectivity of the cell monolayers. The effects of C2 toxin were accompanied by a time- and dose-dependent increase in ADP-ribosylatin of G-actin. G-Actin content increased and F-actin content decreased time- and dose-dependently in C2 toxin-treated endothelial cells. Phalloidin which stabilizes filamentous actin prevented the effects of botulinum C2 toxin on endothelial permeability. Botulinum C2 toxin induced interendothelial gaps. The effects occurred in the absence of overt cell damage and were not reversible within 2 h. The data suggest that the endothelial microfilament system is important for the regulation of endothelial permeability. (*J. Clin. Invest.* 1991. 87:1575–1584.) Key words: cultured pulmonary endothelial cells • hydraulic conductivity • membrane selectivity • cytoskeleton

Introduction

The acute respiratory distress syndrome of the adults is characterized by a noncardiogenic pulmonary edema (1). The mechanisms of increased vascular permeability, however, are poorly understood. Already 20 years ago Majno et al. (2) provided evidence that inflammatory agents induce enhanced vascular permeability in vivo by causing endothelial cell contraction and intercellular gap formation. This concept has been extended to more recently discovered inflammatory mediators such as leukotriene E_4 (3). Endothelial cell retraction was also demonstrated in vitro using cultured endothelial cell monolayers after addition of different inflammatory agents (histamine, bradykinin, thrombin, reactive oxygen species, bacterial toxins) (4–13). Circumstantial evidence suggests that enhanced endothelial permeability is related to alterations of the cellular cytoskeleton (6, 7, 14–16). Endothelial cells have been shown to contain an elaborated microfilament system, consisting of actin, myosin, alpha-actinin, tropomyosin, spectrin, and other components (17–23). Recent studies in skinned endothelial monolayers using *N*-ethylmaleimide modified myosin subfragment 1 suggest that an interaction of endothelial actin and myosin is important for the regulation of endothelial permeability (24). Moreover, endothelial cell retraction appears to be dependent on myosin-light chain kinase phosphorylation of myosin light chains (25). Additional evidence for a role of the endothelial cytoskeleton in regulating the endothelial permeability comes from studies with the microfilament disrupting substance cytochalasin D (26). This agent was shown to reversibly increase the permeability of endothelial cell monolayers. This effect was accompanied by gap formation between cells and dearrangement of endothelial actin filaments (26).

Botulinum C2 toxin is a binary toxin and consists of two components, C2I and C2II (27, 28). Whereas C2I (M_r 50,000) possesses ADP-ribosylating activity (27), C2II (M_r 100,000) appears to be involved in the binding of C2I to the eukaryotic cell surface and the transfer into the cell (28). Botulinum C2 toxin ADP-ribosylates in arginine 177 nonmuscle actin but not skeletal muscle actin (27, 29–31). The toxin modifies actin in its monomeric G-form, while polymerized F-actin is a poor substrate (27). ADP-ribosylation inhibits the ability of actin to polymerize (27, 29). Furthermore, this modification turns actin into a capping protein which binds to the barbed end of actin filaments thereby inhibiting the polymerization of unmodified actin (27, 32). The specific action of botulinum C2 toxin on actin qualifies the toxin as a new tool to study the role of actin in various cell functions (33).

Using botulinum C2 toxin we started to probe the role of actin in the regulation of the permeability of endothelial monolayers in a well-defined in vitro system (12).

Methods

Materials. Tissue culture plasticware was obtained from Becton-Dickinson, Heidelberg, FRG. Medium 199, FCS, HBSS, trypsin-EDTA-solution, Hepes, and antibiotics were from Gibco, Karlsruhe, FRG. Collagenase (type CLS type II) was purchased from Worthington Biochemical Corp., Freehold, NJ. Gelatin from porcine skin type I, calf thymus DNA (type 1), DNAse (type 4), bovine muscle actin, glutaraldehyde grade II, cytochalasin D (CD), thymidine, leukopeptin, phalloidin, and PMSF were obtained from Sigma Chemical Co., Munich, FRG. Polycarbonate micropore filter membranes (25 mm diameter, 5 μm pore size) were purchased from Nuclepore GmbH, Tübingen, FRG. ^3H_2O (1 mCi/g) was supplied by New England Nuclear, Dreieich, FRG, and methyl-[^{14}C]-albumin (0.026 mCi/mg) and [^{32}P]-NAD were from Amersham Buchler, Braunschweig, FRG. Rhodamine-labeled phalloidin was obtained from Molecular Probes, Inc., Junction City, OR. All other chemicals used were analytical grade and obtained from commercial sources.

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Botulinum C2 toxin was prepared and activated essentially as described (28).

Pulmonary artery endothelial cells were obtained from freshly slaughtered pigs by exposure to 0.1% collagenase for 12–15 min (34–36). Endothelial cells were isolated, characterized, maintained, and dispersed as previously described (37–39).

Endothelial cell monolayer on polycarbonate filter membranes. Polycarbonate filter membranes were coated with gelatin, exposed to glutaraldehyde, and sterilized as previously described (12). The coated filter membranes were placed in the bottom of a petri dish, about 2×10^6 cells were seeded on the filter in medium 199/10% FCS and allowed to adhere for 3 h. Additional 10 ml of medium were then added to the petri dish. Thereafter, the monolayers were fed every other day and used ~ 6 d after plating.

Determination of hydraulic conductivity. A confluent filter membrane was mounted in a modified chemotaxis chamber. The upper and lower compartments were filled with HBSS supplemented with 0.25% albumin. One port to the upper compartment is for substitution of filtrated volume and for application of pressure and one port is for the addition of reagents. The lower compartment of the system is part of a semiclosed perfusion system (total volume, 10 ml). A roller pump provides a flow of 10 ml/min. The circulating fluid drips into a 1-ml capillary; the height of the fluid level in this capillary directly corresponds to the volume filtrated through the cell monolayer. A filtrated volume of 10 μl can reliably be measured. The entire system is kept at 37°C by a heated water bath (12). A hydrostatic pressure of 10 cm H_2O was continuously applied to the upper side of the cell monolayer. The filtration rate across the endothelial monolayer was continuously determined (12).

Selectivity of the endothelial cell monolayer. For calculation of this parameter 100 nCi ^3H_2O and 100 nCi [^{14}C]-albumin were added to the upper compartment. The amount of ^3H_2O and [^{14}C]-albumin in the lower compartment was continuously measured in a Ramona LS-5 radioactivity monitor (Raytest, Heidelberg, FRG) consisting of a flowthrough cell, a splitter/mixer, and an IBM-PC for data calculation (for details see reference 12). The endothelial cell monolayer selectivity (MS) was calculated every 5 min on the basis of ^3H_2O and [^{14}C]-albumin in the upper and lower compartments of the filter system. Calculations were done as follows (see also reference 12): $MS = 1 - (A/B \times D/C)$. A and C are the differences (in counts per minute per milliliter) of [^{14}C]-albumin and ^3H_2O, respectively, in the lower compartment of the system between time points x and $x + 5$ min, multiplied by the volume of the lower compartment. B and D are the sums (in counts per minute per milliliter) of [^{14}C]-albumin and ^3H_2O, respectively, in the upper compartment at time points x and $x + 5$ min, divided by 2 (12). Calculation of A/B yields the albumin clearance; calculation of C/D yields the water clearance.

The clearance of albumin or water consists of diffusive and convective parts. With respect to actual volume of fluid transferred from upper to lower compartments diffusion predominates in resting sealed endothelial cell monolayers. The ratio convection to diffusion is 1:4 when hydraulic conductivity is 0.5×10^{-5} cm $\times$ s^{-1} $\times$ cm H_2O^{-1}.

The data calculated according to $1 - (A/B \times D/C)$ do not represent true albumin reflection coefficients because by the experimental approach used it is not known with certainty that, with respect to albumin transport, convection always predominates over diffusion. The equation used yields an index of membrane selectivity. Therefore, this parameter is called the selectivity of the endothelial cell monolayer.

ADP-ribosylation assay. ADP-ribosylation was performed essentially as described (27, 30, 40). Briefly, endothelial cell monolayers were incubated in HBSS (supplemented with 25 mM Hepes and 0.25% BSA) with different concentrations of C2 botulinum toxin for 60 min or with 50 ng/ml botulinum C2I and 50 ng/ml botulinum C2II toxin for the indicated periods of time. Thereafter, cells were washed thrice and lysed in a medium containing 10 mM triethanolamine-HCl (pH 7.5), 0.5 mM PMSF, 0.1 μg/ml leukopeptin, and 2.5 mM EDTA. Then the preparation was frozen, thawed, homogenized, and finally used in the ADP-ribosylation assay. ADP-ribosylation of endothelial lysates was carried out in a buffer containing 10 mM thymidine, 0.5 mM ATP, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 5 μM [^{32}P]-NAD (~ 5 μCi), 1 μg/ml botulinum C2 toxin component I, and 50 mM triethanolamine-HCl, pH 7.4. The reaction was started by the addition of 50 μl of the endothelial lysate to give a total volume of 100 μl. Incubation was carried out for 1 h at 37°C. The reaction was stopped by the addition of 1 ml ice-cold TCA (20% wt/vol), and the radioactively labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis according to the procedure of Laemmli (41). Gels were stained with Coomassie Blue, destained, and exposed to Kodak X-OMAT film for 24–36 h. For quantitative determination of the incorporated [^{32}P]-ADP-ribose the reaction was terminated by the addition of 400 μl of a solution containing 2% SDS (wt/vol) and 1 mg/ml BSA and precipitating the proteins with 500 μl of 30% (wt/vol) TCA. Thereafter, the precipitated proteins were collected onto nitrocellulose filters. The filters were washed 10 times with 2 ml of 6% TCA, placed in scintillation fluid and counted for retained radioactivity.

Measurement of actin filament content. F-Actin was measured as described by Wysolmerski and Lagunoff (15). Endothelial cells were grown in T25-tissue culture flasks, washed thrice with buffer A (pH 7.2, 75 mM KCl, 3 mM $MgSO_4$, 1 mM EGTA, 0.2 mM dithiothreitol, 10 mM imidazole, 10 μg/ml aprotinin, 0.1 mM PMSF), and permeabilized with 0.03% saponin in puffer A for 10 min at room temperature. Cell monolayers were fixed in freshly prepared 3% formaldehyde in buffer A for 20 min at room temperature, washed, and stained in the dark with 0.175 μg/ml rhodamine phalloidin for 30 min. After another wash extraction of rhodamine phalloidin was initiated by addition of ice cold HPLC-grade methanol and continued overnight at −20°C. Methanol extraction resulted in a quantitative removel of phalloidin bound to actin. Endothelial cells in methanol were removed from the flasks with a rubber policeman. The methanol suspension was aspirated, and centrifuged at 8,000 *g*. The rhodamine in the supernatants was determined using an Aminco-Bowman spectrophotofluorometer (Colora, Lorch, FRG). Excitation and emission wavelengths were 542 and 563 nm. Matched cell monolayers were washed with puffer A, lysed with 0.1% triton X-100, scraped from the plate, and used for determination of protein according to Bradford (42). Data were expressed as nanograms phalloidin per microgram cell protein. F-Actin was removed from permeabilized endothelial cells by incubating monolayers in 0.3 M KI overnight at 4°C. After KI incubation monolayers were processed as outlined above. KI extraction resulted in a quantitative removal of endothelial actin.

Measurement of G-actin content. G-Actin was determined by the DNAse inhibition assay of Blikstad et al. (43) as described by Hinshaw et al. (44). 80 μg/ml calf thymus DNA was dissolved in 0.1 M Tris-HCl (pH 7.5), 4 mM $MgSO_4$, and 1.8 mM $CaCl_2$ by gentle stirring at 4°C for 48 h. DNAse 1 stock solution was prepared by adding 10 mg/ml DNAse to 0.125 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM NaN_3, and 0.1 mM PMSF. Working solutions were prepared by 100-fold dilution in 20 mM imidazole (pH 7.5), 30 mM NaCl, and 15% glycerol. Toxin-treated endothelial cells in T-25 flasks were lysed in 500 μl HBSS containing 1% triton X-100, 2 mM $MgCl_2$, 2 mM EGTA, 0.2 mM ATP, 0.5 mM DTT, and 0.1 mM PMSF (lysate solution). G-Actin was measured by mixing in a cuvette 10 μl cell lysate and 10 μl of DNAse solution to 3 ml of DNA solution for 5 s. Absorbance at 260 nm was determined immediately for 55 s in a Kontron-photometer and plotted by using the Kontron-enzyme-kinetic software. A standard curve for 30–70% inhibition of DNAse 1 was obtained by measuring the absorbance after addition of 0.1–2 μg bovine muscel actin. Data were expressed as % G-actin of total cellular protein.

Visualization of F-actin. Because actin is concentrated along the margins of endothelial cells we used rhodamine-labeled phalloidin to visualize interendothelial gap formation. Monolayers were fixed at room temperature for 5 min at a hydrostatic pressure of 10 cm H_2O in HBSS (pH 7.4) containing 2% formaldehyde. After three washes with HBSS monolayers were permeabilized by exposure to acetone at −20°C for 1 min. Cells were then washed with HBSS and incubated for 30 min with rhodamine-labeled phalloidin (1.4 μg/ml). After a brief

wash the preparations were mounted in 60% glycerol (containing 1.5% *n*-propyl gallate) and examined by fluorescence microscopy as previously described (17, 24).

Release of lactate dehydrogenase (LDH) was used as a marker of cytotoxicity. Endothelial cell monolayers grown on the polycarbonate filter membranes were exposed to the botulinum C2 toxin for 2 h. The medium was removed and centrifuged with 8,000 *g* for 2 min. LDH activity in the supernatant was determined by the colorimetric measurement of the reduction of sodium pyruvate in the presence of NADH (45). Enzyme release was expressed as the percentage of total enzyme activity released from endothelial cells in the presence of 100 μg/ml mellitin (12, 46).

Experimental protocol. In this study "sealed" endothelial cell monolayers were used, i.e., monolayers which showed a final filtration rate of < 20 μl/min and a membrane selectivity > 0.65 at a hydrostatic pressure of 10 cm H_2O. Control filters were stable throughout the experimental time (usually 2 h). At the end of the experiments control cells always received 10 μg/ml staphylococcal alpha-toxin (12) to demonstrate the viability and reactivity of the cell monolayers studied.

Aliquots of C2 toxin (in buffer) or of CD (dissolved in DMSO, no independent effect of the solvent) were carefully added to the upper compartment (bolus addition). The injection procedure itself was without effect on the monolayer system as checked by frequent addition of stimulus-free buffer or solvent. In all experiments the components I and II of C2 toxin were added in a ratio of 1:1.

In some experiments the effects of a bolus addition of CD were compared to its continuous application. To study an interaction of both agents, we added submaximal concentrations of C2 toxin and CD simultaneously to the cell monolayers.

Phalloidin (10^{-6} and 10^{-7} M) or the solvent ethanol (0.1 and 0.01%) were added to cell monolayers on filter membranes in complete medium for 16 h. During this period some phalloidin was taken up by endothelial cells as demonstrated by enhanced association of rhodamine-labeled phalloidin with cellular actin (not shown). Phalloidin-uptake by endothelial cells was very slow; preliminary studies indicated that an incubation period of 2 and 4 h was insufficient to load endothelial cells with phalloidin.

Statistical methods. Depending on the number of groups (*A*) and the number of different time points studied (*B*) data of Figs. 1, 2, 6–10 were analyzed by an $A \times B$ analysis of variance. Main effects were then compared by an *F* probability test. When the analysis of variance had shown an interaction, individual data points were compared by Scheffe's test (47).

Results

Addition of components I and II (ratio 1:1) of botulinum C2 toxin to endothelial cell monolayers cultured on a polycarbonate filter membrane resulted in a dose-dependent (3–100 ng/ml) and time-dependent increase in the hydraulic conductivity of the cell monolayers (Fig. 1). Using 100 ng/ml C2 toxin the effect was evident after 25–30 min; 3 ng/ml C2 toxin increased the permeability of endothelial monolayers after ~ 90 min. Control cell monolayers were stable throughout the experimental period and reacted appropriately upon addition of staphylococcal alpha-toxin (Fig. 1) (12).

Both components of the C2 toxin had to be present to induce the increase in endothelial permeability. Addition of 100 ng/ml of toxin component I only or of 100 ng/ml of toxin component II only was without effect on endothelial permeability (data not shown).

Botulinum C2 toxin did not injure endothelial cells; i.e., there was no enhanced release of LDH from toxin-exposed endothelial cells (data not shown). In contrast to cytochalasin

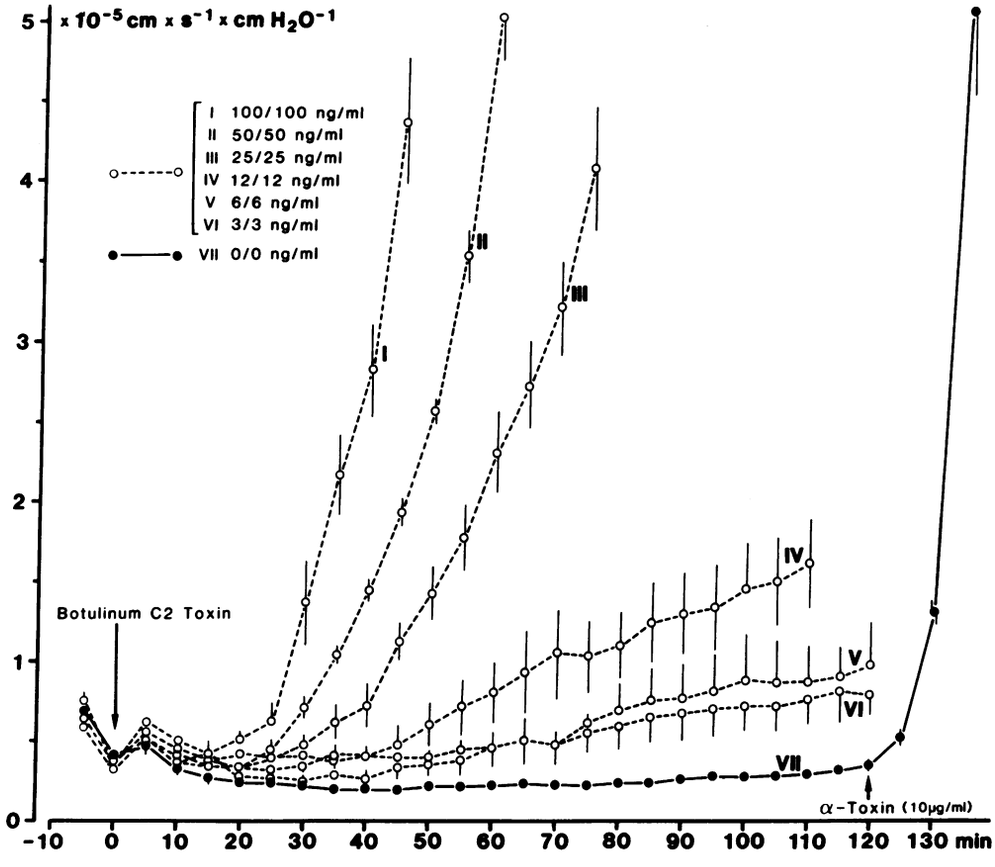

Figure 1. Dose- and time-dependent increase in hydraulic conductivity of endothelial cell monolayer by botulinum C2 toxin. Components I and II of C2 toxin were added in equal amounts. Even the lowest toxin concentration tested (3 ng/ml) significantly increased the water filtration rate across the monolayer. Control monolayers were stable throughout the experimental period and responded promptly upon addition of staphylococcal alpha-toxin. Data presented are mean±SE of seven separate experiments.

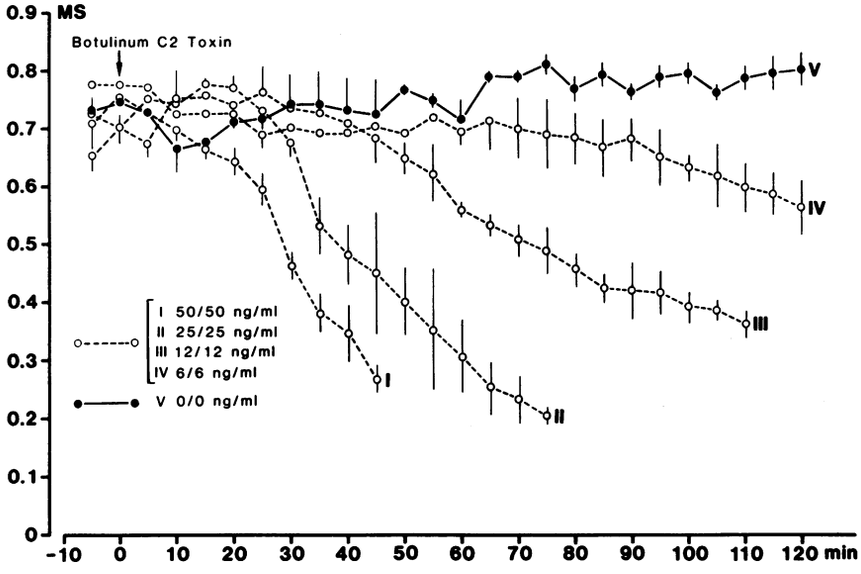

Figure 2. Dose- and time-dependent decrease of the selectivity of endothelial cell monolayers by botulinum C2 toxin. Hydraulic conductivity (Fig. 1) and selectivity (see Methods for experimental details) were determined simultaneously on the same endothelial cell monolayer.

D (see later) the effects of C2 toxin were not reversible within 2 h.

Botulinum C2 toxin also dose-dependently altered the selectivity of endothelial cell monolayers (Fig. 2). In control monolayers this parameter was stable at ~ 0.7 throughout the entire experimental period. It dropped from 0.7 to 0.2 in the presence of 50 ng/ml C2 toxin, i.e., the endothelial cell monolayer lost its permselectivity. Lower C2 concentrations had intermediate effects on the selectivity of the cell monolayers (Fig. 2). Hydraulic conductivity (Fig. 1) and selectivity (Fig. 2) were determined on the same endothelial cell monolayers.

Staining of the endothelial actin filament with rhodamine-labeled phalloidin showed formation of gaps between endothelial cells after exposure to 50 ng/ml C2 toxin for 60 min (Fig. 3).

The C2 toxin–induced alteration of endothelial monolayer permeability was paralleled by an ADP-ribosylation of endothe-

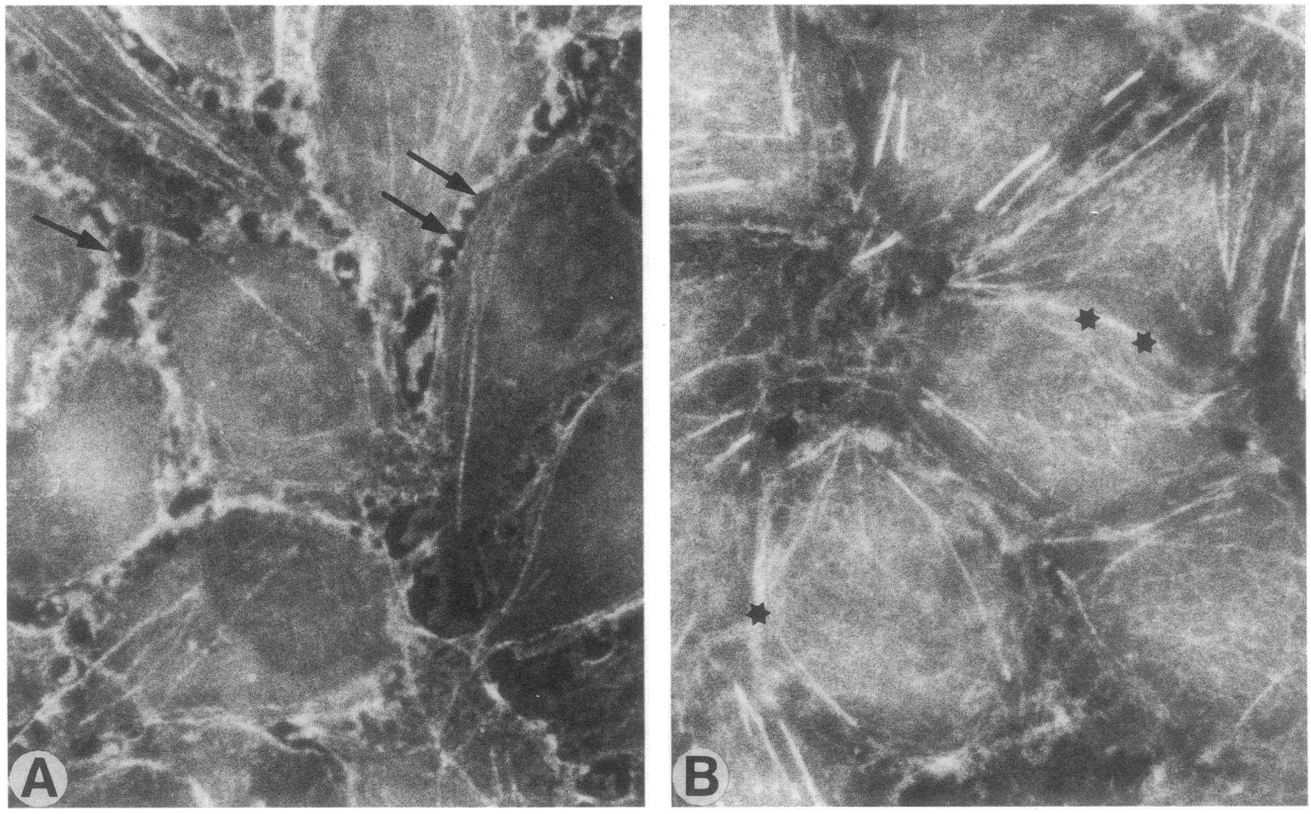

Figure 3. Visualization of interendothelial gaps after staining of endothelial actin filaments with rhodamine-labeled phalloidin. (*A*) Formation of interendothelial gaps (*arrows*) is noted in endothelial cell monolayers exposed to 50 ng/ml botulinum C2 toxin for 60 min, while (*B*) no gaps are seen in control monolayers (*), ×130).

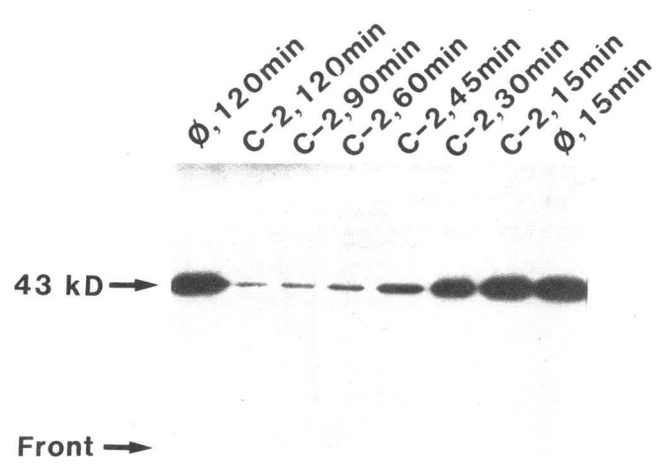

Figure 4. Time-dependent increase in ADP-ribosylation of G-actin (43 kD) in endothelial cell monolayers exposed to botulinum C2 toxin. Endothelial cells were exposed to 25 ng/ml C2 toxin for the indicated periods of time. Cells were then washed and lysed, and the lysates were ADP-ribosylated with 1 μg/ml component I of C2 toxin in the presence of 5 μCi [^{32}P]NAD as outlined in Methods. Radioactively labeled proteins were analyzed by SDS-PAGE and an example of the autoradiograms of the gels is shown.

lial actin which was determined in cell lysates by the reduction of a second C2I toxin–induced ADP-ribosylation of actin with [^{32}P]-NAD. The autoradiogram in Fig. 4 illustrates that in endothelial lysates only one protein with 43 kD, corresponding to G-actin, was ADP-ribosylated by C2 toxin. ADP-ribosylation of endothelial actin occurred in a time and concentration dependent manner (Figs. 4, 5). As shown in Fig. 1, treatment of endothelial cells with 25 ng/ml C2 toxin for 30 min elicited a

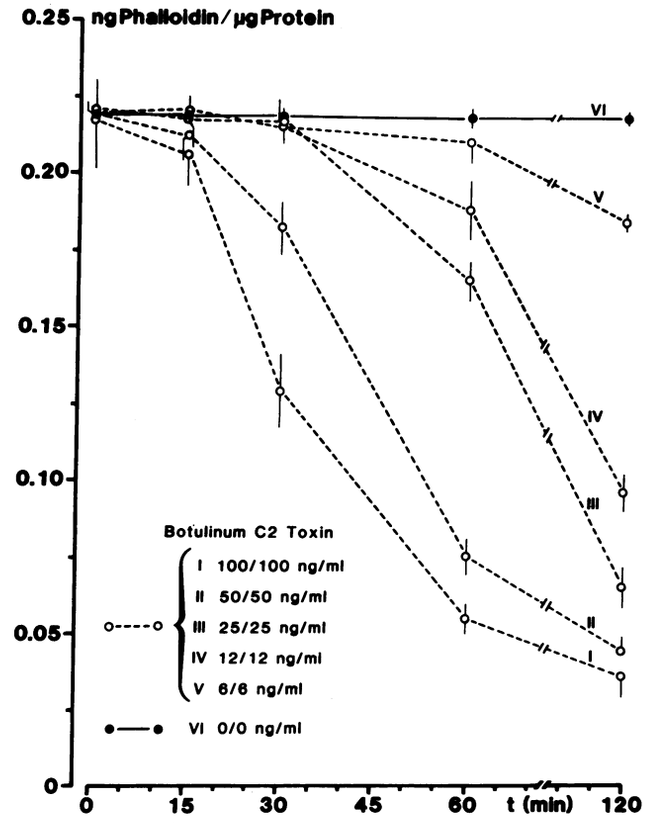

Figure 6. Time- and dose-dependent decrease in endothelial F-actin content by botulinum C2 toxin. This parameter was determined by quantitating actin-bound rhodamine phalloidin as outlined in Methods. Data presented are mean±SE of seven separate experiments.

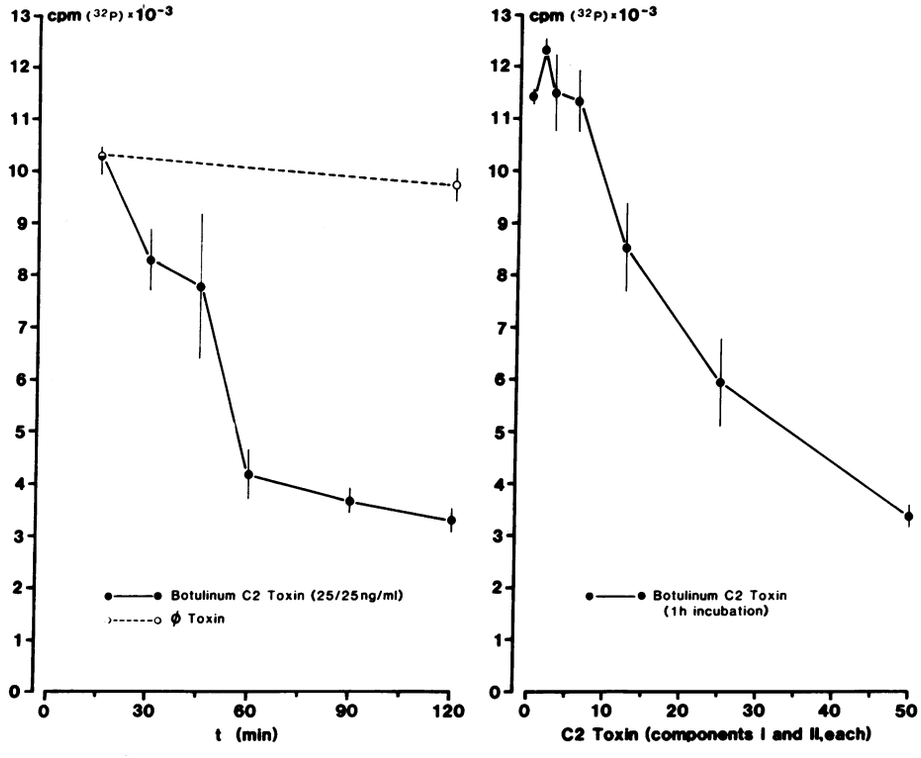

Figure 5. Time-dependent (*left*) and dose-dependent (*right*) increase in ADP-ribosylation of G-actin (43 kD) in endothelial cell monolayers exposed to botulinum C2 toxin. Cells were treated with 25 ng/ml C2 toxin for the indicated periods of time (*left*) or for 1 h with the indicated concentrations of C2 toxin (*right*). For details see legend of Fig. 3. Data presented are mean±SE of three separate experiments.

significant increase in permeability. After this period of time ~ 20% of the modifiable actin was ADP-ribosylated by the toxin (Fig. 5 *A*). Studies on the concentration dependency of C2 toxin–induced ADP-ribosylation revealed that in endothelial cells 12 ng/ml of C2 toxin was the lowest concentration to induce a significant ADP-ribosylation of actin after 1 h (Fig. 5 *B*). Identical toxin concentrations were necessary for a significant increase in endothelial permeability (Fig. 1).

In endothelial cell monolayers botulinum C2 toxin induced a dose- and time-dependent increase in G-actin content (Fig. 6) and decrease in F-actin content (Fig. 7). As shown in Fig. 1, for a 60-min incubation 12 ng/ml C2 toxin was the lowest concentration to increase endothelial permeability. After this period of time G-actin content had increased by ~ 15%. At the same time F-actin content had decreased to a similar extent.

Next we examined the effects of C2 toxin on endothelial permeability in the presence of an agent which stabilizes F-actin, i.e., phalloidin which decreases the rate of actin depolymerization (48, 49). Endothelial cell monolayers were incubated with 10^{-6} and 10^{-7} M phalloidin overnight, washed several times, and mounted into the chambers. In phalloidin-pretreated cell monolayers the increase in endothelial permeability following C2 toxin was reduced by 90% (Fig. 8). Phalloidin at the concentrations used was not toxic to endothelial cells as

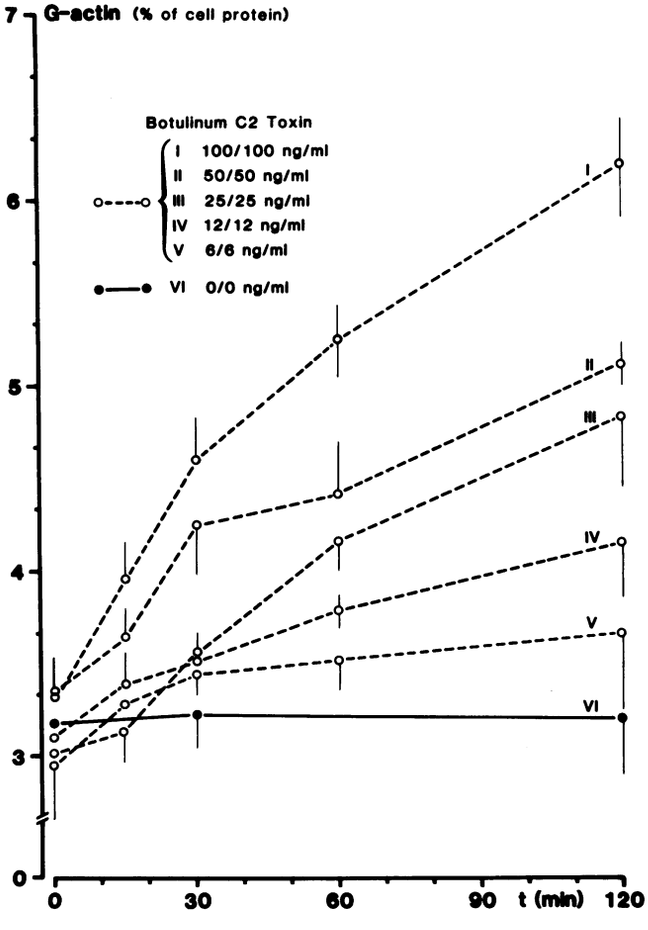

Figure 7. Time- and dose-dependent increase in endothelial G-actin content by botulinum C2 toxin. This parameter was determined using the DNAse inhibition assay as outlined in Methods. Data presented are mean±SE of four separate experiments.

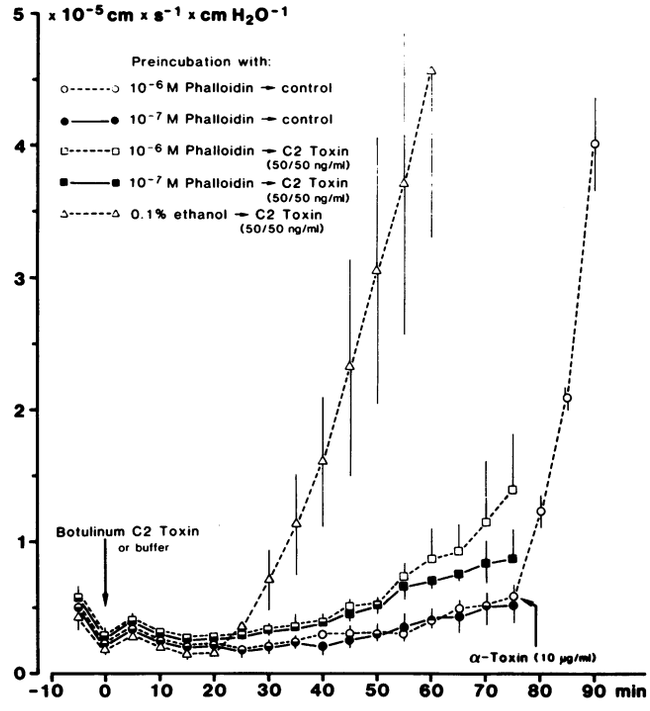

Figure 8. Reduction of the C2 toxin–induced increase in the hydraulic conductivity of endothelial monolayers by pretreatment of cells with 10^{-6} and 10^{-7} M phalloidin for 16 h. Phalloidin pretreatment, however, did not impair the effect of staphylococcal alpha-toxin. Different groups were studied simultaneously. Data presented are mean±SE of six separate experiments.

indicated by no enhanced LDH-release (not shown). In addition, phalloidin-pretreated monolayers showed a normal basal permeability and reacted promptly upon addition of staphylococcal alpha-toxin (Fig. 8) (12). Moreover, phalloidin pretreatment did not reduce C2 toxin–related ADP-ribosylation: incorporated [^{32}P]-ADP-ribose in endothelial cells exposed to 0, 25/25 ng/ml, and 50/50 ng/ml C2 toxin were 11,400±763, 6,150±325, and 3,290±290 cpm; the corresponding data for phalloidin-treated cells were 10,820±490, 5,750±473, and 3,660±140 cpm (mean±SE, n = 3).

We then compared the effects of C2 toxin with those of cytochalasin D (CD),[1] an agent which caps barbed ends of actin filaments and presumably possesses filament severing activity (50–53). Bolus addition of CD in the range of 0.5–1.0 µg/ml dose-dependently increased the hydraulic conductivity of endothelial cell monolayers. The CD effect peaked at 10 min and then steadily declined within 60–90 min (Fig. 9 *A*). Thus, in contrast to C2 toxin, the effects of CD appeared to be reversible. To study this aspect in more detail, a bolus addition of CD was compared to a continuous application (*a*) and the effects of CD were studied in an on-off-on-fashion (*b*) (Fig. 9 *B*): (*a*) A continuous application of CD, thus maintaining 0.75 µg/ml CD in the upper compartment of the filter system, induced a rapid and large increase in the permeability of the endothelial monolayers which was higher and which did not decline as compared to a bolus addition of 0.75 µg/ml CD. (*b*) Changing

1. *Abbreviation used in this paper:* CD, cytochalasin D.

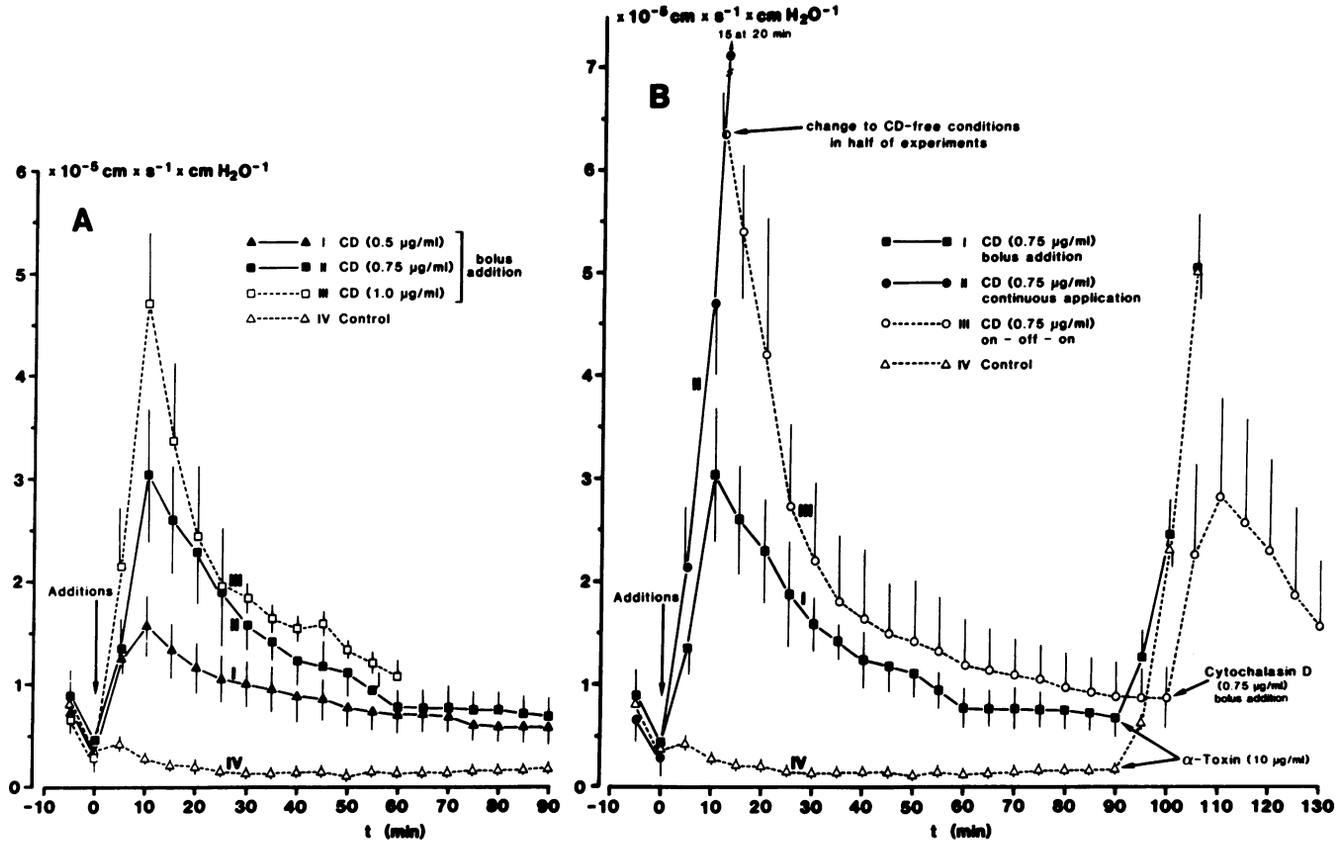

Figure 9. Influence of cytochalasin D (*CD*) on the hydraulic conductivity of endothelial cell monolayers: (*A*) Dose-dependent reversible increase in endothelial permeability upon bolus addition of cytochalasin D (*CD*) at time point 0. Data presented are mean±SE of six separate experiments. (*B*) Comparison of continuous versus bolus application of CD. Upon addition of CD as a bolus the increased permeability peaked at 10 min and then steadily declined (I). A continuous application of CD resulted in a large and continuous increase in endothelial permeability (II). Change to CD-free conditions reversed the effect (III). Readdition of CD raised again endothelial permeability. Staphylococcal alpha-toxin rapidly increased endothelial permeability in control cells and cells previously exposed to CD. Data presented are mean±SE of four separate experiments.

the fluid in the upper compartment to CD-free conditions reversed the CD-induced increased endothelial permeability; readdition of 0.75 µg/ml CD restored the effect again (Fig. 9 *B*).

Additional studies showed a rapid decrease in endothelial F-actin content after continuous exposure to 0, 0.5, 0.75, and 1.0 µg/ml CD. After 2 min F-actin content (indicated as nanograms phalloidin/milligram protein) dropped from 265±3 to 257±5, 245±5, and 217±4 (mean±SE, n = 7).

Finally, the combined effects of submaximal concentrations of C2 toxin and CD on endothelial monolayer permeability were studied. 12.5 ng/ml C2 toxin alone and 0.5 µg/ml CD alone had moderate effects on the permeability of the endothelial monolayer. Addition of both agents (12.5 ng/ml C2 toxin, 0.5 µg/ml CD) resulted in a synergistic increase of endothelial permeability (Fig. 10). Interestingly, the kinetics of each agent were preserved. There was, however, a merely additive effect of both agents on endothelial F-actin content (data not shown).

Discussion

This study demonstrates that botulinum C2 toxin which ADP-ribosylates actin, causes an increase in the permeability of endothelial cell monolayers derived from porcine pulmonary arteries. With the highest toxin concentration used (100 ng/ml) the hydraulic conductivity increased 10-fold. In addition, the cell monolayer lost its permselectivity as indicated by the large drop of the index of selectivity from 0.7 to 0.2. Even 3 ng/ml toxin significantly increased endothelial permeability within 2 h. The changes in permeability were accompanied by formation of intercellular gaps between endothelial cells thereby possibly opening a paracellular pathway for enhanced fluid exchange. C2 toxin–induced enhancement of endothelial monolayer permeability occurred in the absence of overt cell damage and was not reversible within 2 h.

In this study endothelial permeability was investigated on "sealed" endothelial cell monolayers in the presence of a well-defined transendothelial pressure gradient. Previously we have shown that application of a continuous hydrostatic pressure resulted in a "sealing" of endothelial cell monolayers, i.e., within 1–2 h the hydraulic conductivity decreased and the permselectivity increased (12, 46). We believe that this procedure, as compared to studies done under strict diffusion conditions, will allow a better simulation of the in vivo situation.

The effects of C2 toxin on endothelial permeability were accompanied by an increased ADP-ribosylation of endothelial G-actin. Recently it was demonstrated that C2 toxin-induced ADP-ribosylation of G-actin causes depolymerization of F-actin and an increase in the G-actin pool (30, 54). Assuming that

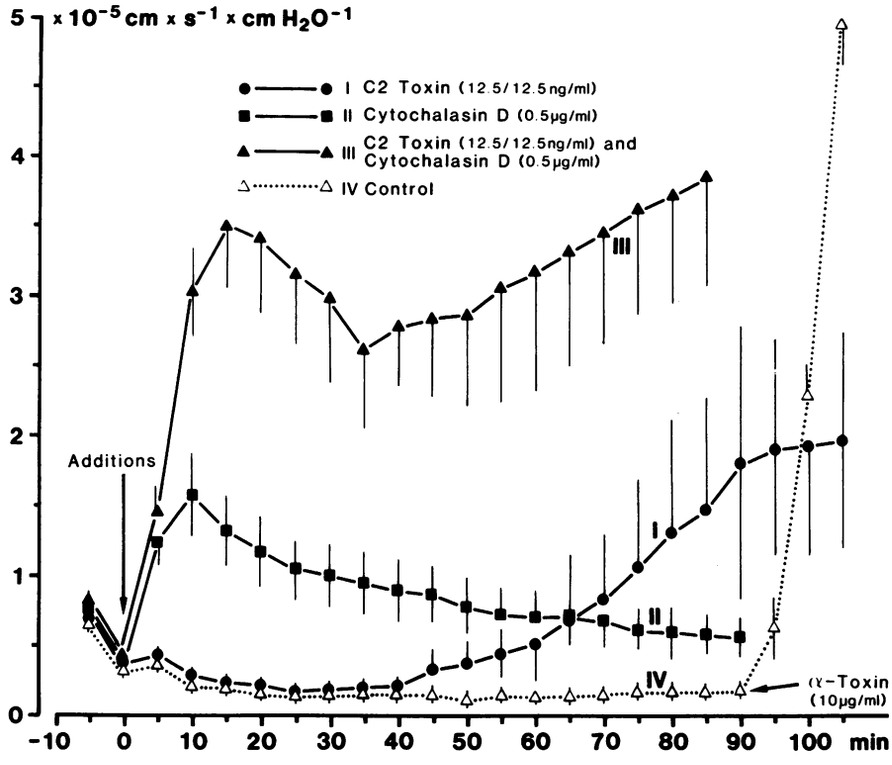

Figure 10. Synergistic effect of CD and C2 toxin on endothelial permeability. Submaximal concentrations of CD (0.5 µg/ml) or C2 toxin (12.5 ng/ml) had a moderate effect on the permeability of an endothelial cell monolayer while a combination of both markedly increased it. Experiments were performed simultaneously. Data presented are mean±SE of six separate experiments.

in resting endothelial cells a dynamic equilibrium exits between polymerized and nonpolymerized actin botulinum C2 toxin alters this balance in favour of increased actin depolymerization. This point was proven experimentally in the present study by quantitating F- and G-actin content. G-Actin increased and F-actin decreased in C2 toxin–treated endothelial cells in a time- and dose-dependent manner. Because ADP-ribosylated actin lost its ability to polymerize C2 toxin–induced ADP-ribosylation reduces the effective concentration of the actin monomer pool participating in filament growth (33). Moreover, toxin-modified actin acts like a capping protein thereby preventing further addition of G-actin to the growing end of F-actin (32). In contrast, ADP-ribosylation of actin does not block or reduce depolymerization at the pointed end of actin filaments.

The notion that C2 toxin–induced actin depolymerization was involved in enhanced endothelial permeability was supported by studies using phalloidin. This agent binds tightly to polymerized actin and shifts the equilibrium between actin filaments and actin monomers towards filament formation largely by decreasing the rate of the actin depolymerization (48, 49). The C2 toxin–induced increase in endothelial permeability was substantially reduced in phalloidin-loaded cell monolayers suggesting that phalloidin prevents the C2 toxin–induced imbalance between actin polymerization and depolymerization.

Phalloidin does not readily permeate cell membranes and its uptake in cells (with the exception of hepatocytes) probably proceeds by pinocytosis (50). In the present study endothelial cells had to be incubated with phalloidin for at least 16 h in order to obtain a phalloidin-loading which was sufficiently high to block the effects of C2 toxin. In addition, after 16 h an increased staining of cells with rhodamine-labeled phalloidin was noted by immunofluorescence. A recent study showed that phalloidin reduced the histamine- and bradykinin-induced increase in permeability of bovine aortic endothelial cells (55), supporting the view that phalloidin can enter endothelial cells.

It is of interest that phalloidin pretreatment did not block the increase in the permeability of endothelial cell monolayers by staphylococcal alpha-toxin. Exposure of endothelial cells to staphylococcal alpha-toxin, a channel forming protein (56), will increase intracellular free Ca^{2+}. Recently, we have hypothesized that this may trigger an interaction between actin and myosin which finally would result in an active retraction of cells with subsequent formation of intercellular gaps (12, 24). Based on these data it is conceivable that phalloidin pretreatment did not impair the endothelial response to staphylococcal alpha-toxin.

Another aspect of interest is that in the present study phalloidin pretreatment did not reduce the permeability of resting endothelial monolayers. This is in contrast to Alexander et al. (55) who noted a decreased basal endothelial permeability in phalloidin-treated cells. In the present study resting cells probably had already optimized their barrier function which could not be further improved by increasing F-actin.

Studies using CD were performed to corroborate the notion that the microfilament system is important for regulating endothelial monolayer permeability. The most prominent effect of CD is its capping function, i.e., its reversible binding to the barbed end of actin filaments; in addition, CD reportedly severs F-actin (50–53). In our system CD caused a dose-dependent increase in endothelial permeability which was readily reversible upon changing to CD-free conditions and which could be reproduced several times within the same cell monolayer. These studies confirm and extend experiments by Shasby et al. (26).

All these data presented can be interpreted to indicate that an intact endothelial microfilament system is important for maintaining a functionally competent endothelial barrier. It is

not clear, however, how ADP-ribosylation of G-actin and depolymerization of actin result in increased permeability of endothelial cell monolayers. It should be emphasized that the permeability of an endothelial monolayer is most likely not simply governed by the ratio of F- to G-actin pools. Several observations presented herein (a small decrease/increase in F-/G-actin content altered endothelial permeability; studies combining CD and C2 toxin) suggest that polymerization/depolymerization of selective actin pools are involved in the regulation of endothelial permeability. For epithelium, for example, it is known that the microfilament system is associated with tight junctional proteins (57–59). If the same is true for endothelium actin disassembly may have a disorganizing effect on intercellular junctions which in turn may result in the observed cell retraction with subsequent gap formation and enhanced paracellular flux of water and albumin. Thus, the quantitation of the contributions made by selective actin pools (dense peripheral band, central microfilaments, plasma membrane–associated microfilaments) will be of considerable interest.

The interpretation of the data presented is limited especially because the studies were performed on cultured porcine pulmonary artery endothelial cells. For technical reasons it is currently not possible to examine human pulmonary microvascular endothelial cells. Therefore, the applicability of the data presented to the human pulmonary microcirculation is not clear.

The previous evidence for the hypothesis that the cellular microfilament system is involved in the regulation of endothelial permeability was primarily based on alterations of the distribution of F-actin and on studies using cytochalasins, reagents with questionable specificity (50). The findings presented indicate that the specific actin tool botulinum C2 toxin and the controlled endothelial cell monolayer model are apparently useful to elucidate the role of cytoskeletal components for the regulation of vascular permeability.

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