Cytochalasin B modulation of Caco-2 tight junction barrier: role of myosin light chain kinase

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A major function of intestinal epithelial cells is to provide a physical barrier between the hostile intestinal lumen and the subepithelial tissue. The apically located tight junctions (TJs) form a paracellular barrier between the lateral membranes of the adjacent cells and act as a structural barrier against the paracellular penetration of hydrophilic molecules (2, 31). Disruption of the intestinal epithelial TJ complexes results in a “leaky gut” with an increase in intestinal paracellular permeability (18, 25, 31). It had been proposed in some diseases that a defective intestinal epithelial TJ barrier allows the paracellular permeation of toxic luminal substances, which leads to intestinal inflammation and mucosal injury (3, 11, 14, 18, 25, 40). Specifically, evidence had been presented suggesting that an altered intestinal epithelial TJ permeability may be an important pathogenic factor in intestinal diseases such as Crohn’s disease (18, 19, 25, 36), nonsteroidal anti-inflammatory drug-associated enteritis (3), and in diarrheal syndromes caused by Clostridial difficile, Vibrio cholera, and enteropathogenic Escherichia coli (11, 14, 40). The precise intracellular processes that regulate intestinal epithelial TJ permeability in pathological and normal physiological conditions remain poorly understood.

The intercellular TJs encircle the intestinal epithelial cells in a belt-like manner at the apical cellular borders at the level of zonula occludens. The TJs make homotypic contact across the intercellular spaces between the adjacent cells (2). The lateral contacts, which may be visualized by electron microscopy and freeze-fracture analysis, act as a structural barrier against the paracellular permeation (31, 34). There is also a high density of cytoskeletal elements and actin and myosin filaments, which encircle the intestinal epithelial cells near the apical cellular borders at the level of zonula adherens (31–34). Previous studies (29, 33) have shown that disruption of the paracellular actin filaments with cytochalasins (specific actin-disrupting agents) causes an increase in intestinal epithelial TJ permeability. Cytochalasins disrupt actin microfilaments by a direct severing effect, interfering with actin subunit polymerization and inducing reactive cellular response (4, 5, 12, 33, 39). In this regard, cytochalasins have been widely used as probes for studying actin-mediated cell activities.

The cytochalasin disruption of paracellular actin filaments culminates in morphological and functional disturbance of intestinal TJs (29, 33). The intracellular mechanisms that modulate this actin filament-mediated increase in intestinal TJ permeability have not been fully characterized.

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been delineated. Because actin function is closely dependent on its interaction with myosins, we hypothesized that cytochalasin modulation of TJ permeability is mediated by regulation of myosin light chain kinase (MLCK) activity. Specifically, we tested the hypothesis that cytochalasin-induced increase in intestinal TJ permeability was mediated by MLCK activation and perijunctional actin-myosin interaction and that MLCK activation was an important triggering event leading to the increase in intestinal TJ permeability. We used the filter-grown Caco-2 intestinal epithelial monolayers as the in vitro model system to study the effects of Cyto B on intestinal epithelial TJ permeability. The human colon cancer-derived Caco-2 intestinal epithelial cell system has been widely used as an in vitro model of intestinal epithelia (15, 16, 26, 38). Our results provide new insight into the intracellular mechanism of cytochalasin modulation of intestinal epithelial TJ permeability.

MATERIALS AND METHODS

DMEM, trypsin, and fetal bovine serum were purchased from Life Technologies (Gaithersburg, MD). Glutamine, penicillin, streptomycin, and PBS solution were purchased from Irvine Scientific (Santa Ana, CA). Cyto B was purchased from Sigma Chemical (St. Louis, MO). Millicell-HA 0.4-μm permeable filters (12 mm) were purchased from Millipore (Bedford, MA). Anti-ZO-1 antibody and FITC-strepaavidin were obtained from Zymed Laboratories (San Francisco, CA), and fluorescein-conjugated rabbit anti-rat antibodies were obtained from Boehringer Mannheim (Indianapolis, IN). [14C]mannitol was obtained from NEN Research Products (Wilmington, DE). All other chemicals were of a reagent grade.

Cell cultures. Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). The stock cultures were grown in a culture medium composed of DMEM with 4.5 mg/ml glucose, 50 U/ml penicillin, 50 U/ml streptomycin, 4 mM glutamine, and 10% fetal bovine serum (16, 38). Culture medium was changed every 1–2 days. The cells were grown in a culture medium composed of DMEM with 4.5 mg/ml glucose, 50 U/ml penicillin, 50 U/ml streptomycin, and PBS solution purchased from Irvine Scientific (Santa Ana, CA). Caco-2 monolayers were labeled with appropriate primary antibody. This was followed by incubation with 1:30 diluted Tris-buffered saline solution containing secondary anti-rabbit IgG biotinylated antibody (Zymed Laboratories) and incubation with 1:20 diluted Tris-buffered saline solution containing FITC-labeled strepavidin (Zymed Laboratories). Coverslips were washed in 60% glycerol, Tris-buffered saline solution, and 0.4% n-propyl gallate. The fluorescein-labeled structures were viewed by a “blinded” person unaware of the coded experimental conditions, using either Nikon-PCM 2000 confocal imaging system attached to a Nikon Eclipse 800 microscope or Optronics DEI-750 CE digital output imaging system (Goleta, CA) attached to Nikon Labophot epifluorescence microscope. The photomicrographic images of fluorescein-labeled ZO-1 proteins and the perijunctional actin and myosin filaments were obtained at focal levels corresponding to the region of zonula occludens (2–3 μm below the apical brush-border membrane) and zonula adherens (3–4 μm below the apical brush-border membrane), respectively, using the Optronics imaging system. All of the fluorescent labeling experiments were repeated four to six times in duplicates to ensure reproducibility.

Caco-2 MLCK-kinase activity determination. Caco-2 MLCK activity was determined by measuring in vitro kinase activity of the immunoprecipitated MLCK obtained from the Caco-2 cells after treatment with various experimental agents. For MLCK immunoprecipitation, Caco-2 monolayers were serum-deprived overnight. The Caco-2 cells were then exposed to appropriate experimental conditions. At the completion of the experiments, Caco-2 cells were immediately rinsed with ice-cold Hanks’ balanced salt solution. Cells were then lysed using 0.8 ml lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 μM pepstatin, 1 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 2 μg/ml aprotinin, and 40 mM para-nitrophenol phosphate. The filter-grown Caco-2 monolayers reached epithelial resistance of 340–420 Ω·cm² and were serum-deprived overnight. The Caco-2 cells were then exposed to appropriate experimental conditions. At the completion of the experiments, Caco-2 cells were immediately rinsed with ice-cold Hanks’ balanced salt solution. Cells were then lysed using 0.8 ml lysis buffer (50 mM HEPES, 100 mM NaCl, 2 mM EDTA, 1 μM pepstatin, 1 μg/ml leupeptin, 2 mM phenylmethylsulfonyl fluoride, 2 mM sodium vanadate, 2 μg/ml aprotinin, and 40 mM para-nitrophenol phosphate.
di-cyclohexylammonium salt) and scraped, and lysates were placed in Microfuge tubes (tube A) and microfuged for 5 min to yield a clear lysate.

Anti-MLCK antibody (5 μL/200 μL lysis buffer) obtained from Sigma Chemical was added to a separate Microfuge tube (tube B) containing protein A beads and incubated end-over-end for 1 h at 4°C. Then 100 μL of each cleared lysate (tube A) were added to the microvial (tube B) containing the pelleted protein A-Sepharose bead coupled with anti-MLCK antibodies and incubated end-over-end for 2 h at 4°C. The microvial containing the immunoprecipitates was microfuged, and the supernatant was aspirated. Immunoprecipitates were washed sequentially with lysis buffer and a solution of 10 mM HEPES and 10 mM Mg acetate at 4°C.

Immunoprecipitated MLCK was then used in an in vitro kinase reaction in Microfuge tubes to determine the MLCK activity by measuring the rate of MLC phosphorylation by the immunoprecipitated MLCK. For this, 20 μL purified chicken gizzard MLCK protein (2 mg/mL), 20 μL of three times hot mix (150 μM ATP, 10 μL [γ-32P]ATP (5 μCi/reaction), 30 mM magnesium acetate, and 30 mM HEPES) were added and mixed with the immunoprecipitated MLCK, for a 10-min reaction period at 30°C. The MLCK catalyzed phosphorylation reaction was terminated by addition of 20 μL stop buffer solution (1 mL 2 M Tris buffer, pH 6.8, 2 mL 20% SDS, 4 mL glycerol, 3 mL water, 308 mg dithiothreitol, and trace of bromophenol blue). Subsequently, the reaction mixture was boiled for 3 min and microfuged for 10 s, and then the supernatant (40–50 μL) was separated on 10% SDS-PAGE. The gel was fixed in 40% MeOH and 10% acetic acid overnight and stained with Coomassie blue solution, dried, and autoradiographed, and the MLC band at 19.5 kDa was identified. The experiments were repeated three times to ensure reproducibility. It should be noted that similar results were also obtained when Ca\(^{2+}\) (0.2 mM) and calmodulin (1 μM) were added to the kinase reaction mixture.

**Intracellular MLC phosphorylation assay.** After cell-cycle synchronization in serum-free buffer solution overnight, Caco-2 monolayers were incubated for 1 h at 37°C in phosphate-free medium containing 5% dialyzed fetal bovine serum. At the end of the incubation period, monolayers were washed and labeled with [32P]i (final concentration, 0.2 mCi/mL) for 2 h at 37°C. Subsequently, monolayers were exposed to various experimental conditions. At the end of the experimental period, monolayers were washed with iced-cold PBS, then lysed with lysis buffer for 30 min at 4°C. The lysates were microcentrifuged, and MLC was immunoprecipitated from the supernatant with anti-MLC antibody (Sigma Chemical) at 4°C. After centrifugation and rinse, the immunoprecipitated MLC was resolved by SDS-PAGE on a 12% gel, followed by an autoradiography.

**RESULTS**

**Effect of Cyto B on Caco-2 actin filaments and TJ permeability.** The Cyto B effect on Caco-2 actin microfilaments and TJ permeability was determined by fluorescein labeling of Caco-2 actin filaments and measurements of epithelial resistance and mucosal-to-serosal flux of paracellular marker mannitol across the filter-grown Caco-2 monolayers. Consistent with the native intestinal epithelia (32), Caco-2 actin filaments were localized at the apical perijunctional area at the level of zonula adherens just below the zonula occludens and appeared as a continuous band encircling the cells at the cellular borders (Fig. 1A). Cyto B (5 μg/ml) treatment resulted in a progressive disruption of the Caco-2 actin filaments with breakage, displacement and clumping of the perijunctional actin filaments (Fig. 1, B–D). Cyto B (5 μg/ml) treatment resulted in a drop in Caco-2 epithelial TJ permeability (Fig. 2A) and an increase in mucosal-to-serosal flux of mannitol (Fig. 2B), indicating an increase in Caco-2 epithelial TJ permeability.

Cytochalasins disrupt actin filaments by direct breakage of actin filament network as well as through secondary cellular response (39). After initial breakage or severing of actin filaments into smaller fragments, the actin fragments later combine to form large cytoskeletal clumps or “foci” (33, 39). Similarly, in our studies, two distinct time-related alterations in perijunctional actin filaments were visible after Cyto B
The earliest changes, which occurred within the first minute of the Cyto B exposure, were characterized by the breakage of the perijunctional actin filaments into smaller actin fragments (Fig. 1). These early changes were seen diffusely throughout the cytoplasm at the level of zonula adherens, giving a hazy “fluffy” appearance in the cytoplasm with some actin filaments remaining localized at the cellular borders. On longer exposure, the smaller actin fragments coalesced into large actin clumps or foci (Fig. 1, C and D). This “late phase” formation of actin clumps was inhibited by metabolic inhibitors 2,4-dinitrophenol (1 mM) and sodium azide (30 mM) (Fig. 3B). In contrast, the early phase changes in actin filaments (breakage into smaller fragments) were not inhibited by the metabolic inhibitors (Fig. 3A). In fact, there appeared to be a slight accentuation of actin fragment formation after pretreatment with metabolic inhibitors (Fig. 3B).

**Role of MLCK in Cyto B-induced increase in Caco-2 TJ permeability.** In the following studies, we examined the involvement of Caco-2 MLCK in Cyto B-induced increase in Caco-2 TJ permeability. First, the effect of Cyto B on Caco-2 MLCK activity was examined by immunoprecipitation of Caco-2 MLCK. After Cyto B (5 μg/ml) treatment, Caco-2 MLCK was isolated by immunoprecipitation with anti-MLCK antibody. The kinase activity of the immunoprecipitated MLCK was then determined by measuring in vitro MLC phosphorylation. MLCK obtained from the Cyto B-treated cells produced a significant increase in in vitro MLC phosphorylation compared to the control.

![Fig. 2. Effect of Cyto B (5 μg/ml) on Caco-2 epithelial resistance and paracellular permeability. A: Cyto B (5 μg/ml) effect on Caco-2 epithelial resistance expressed as Ω·cm². Inset: magnified view of the early time course. B: Cyto B (5 μg/ml) effect on mucosal-to-serosal flux of paracellular marker mannitol expressed in nmol/cm². Values are means ± SE; n = 4.](image)

![Fig. 3. Effect of metabolic inhibitors on Cyto B induced modulation of perijunctional Caco-2 actin microfilaments (en face views). Caco-2 cells were energy depleted by incubation with 2-deoxy-D-glucose (2 mM) and 2,4-dinitrophenol (1 mM) for 1 h. Subsequently, the energy-depleted Caco-2 monolayers were treated with Cyto B (5 μg/ml) for 1 (A) and 30 min (B), respectively (original magnification, ×80). 2,4-Dinitrophenol did not affect the Cyto B fragmentation of actin filaments at the 1-min time period, but prevented the actin clump formation and further accentuated the actin fragmentation at the 30-min time period. Similar results were also obtained with sodium azide.](image)

![Fig. 4. Effect of Cyto B on Caco-2 myosin light chain kinase (MLCK) activity. Caco-2 MLCK activity was determined as described in MATERIALS AND METHODS. Caco-2 monolayers were exposed to Cyto B for increasing time periods (0–30 min). Subsequently, Caco-2 monolayers were lysed, and Caco-2 MLCK was immunoprecipitated. The activity of the immunoprecipitated MLCK was determined by in vitro kinetic measurement of MLC phosphorylation. Phosphorylated MLC (P-MLC; ~19.5 kDa) was separated by 10% SDS-PAGE, stained with Coomassie blue solution, and autoradiographed as described in MATERIALS AND METHODS. Cyto B produced a time-dependent activation of Caco-2 MLCK with the peak activation occurring between 5 and 10 min after Cyto B exposure.](image)
Phosphorylation compared with that of control or untreated cells, indicating activation of Caco-2 MLCK (Fig. 4). The time course of Cyto B effect on Caco-2 MLCK activation indicated that the peak MLCK activation occurred between 5 and 10 min after Cyto B exposure (Fig. 4). Direct addition of Cyto B to the immunoprecipitated MLCK did not have significant effect on MLCK activity, indicating that Cyto B does not directly activate MLCK under in vitro conditions.

To determine whether Cyto B-induced activation of Caco-2 MLCK was due to an increase in MLCK protein level or an increase in the activity of the preexisting MLCK proteins, the effects of Cyto B on MLCK protein level and cellular localization were examined by Western blot analysis and immunofluorescent antibody labeling. In the confluent Caco-2 monolayers, MLCK was localized mainly at the perijunctional areas (data not shown). Cyto B did not have significant effect on either MLCK protein level (Fig. 5A) or the cellular localiza-

Fig. 5. Effect of MLCK inhibitor 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) on Cyto B activation of Caco-2 MLCK activity and Caco-2 MLCK protein level. Caco-2 monolayers were incubated with either Kreb’s-phosphate saline buffer (control) or Kreb’s-phosphate saline buffer containing either Cyto B (5 μg/ml), Cyto B (5 μg/ml) and ML-7 (15 μM), or ML-7 (15 μM) for 10 min. Subsequently, Caco-2 cells were lysed, and MLCK activity and protein level were determined by Western blot analysis and in vitro kinase activity measurement as described in MATERIALS AND METHODS.

A: Western blot analysis. B: immunoblot of phosphorylated MLC. C: corresponding densitometry measurements (means ± SE) of phosphorylated MLC bands expressed in pixels (n = 4).

Fig. 6. Effect of Cyto B on Caco-2 MLC phosphorylation inside the cells. Intracellular MLC phosphorylation was determined by direct immunoprecipitation of 32P-labeled MLC as described in MATERIALS AND METHODS. The immunoprecipitated MLC was resolved by 12% SDS-PAGE. Cyto B (5 μg/ml) caused an increase in MLC phosphorylation inside the treated cells. ML-7 inhibited the Cyto B-induced increase in MLC phosphorylation.

Fig. 7. Effect of MLCK inhibitors on Cyto B modulation of Caco-2 epithelial resistance and paracellular permeability. A: effect of ML-7 (15 μM) on Cyto B (5 μg/ml)-induced drop in Caco-2 epithelial resistance. B: effect of ML-7 (15 μM) on Cyto B (5 μg/ml)-induced increase in mucosal-to-serosal flux of mannitol. A and B: n = 4.
tion of MLCK (data not shown), suggesting that Cyto B stimulation of Caco-2 MLCK activity resulted from an increased activity of the preexisting MLCK proteins.

The Cyto B effect on Caco-2 MLCK activity was further validated by measurement of MLC phosphorylation inside the cells by direct immunoprecipitation of MLC. The Cyto B treatment produced a significant increase in phosphorylation of MLC inside the cells (Fig. 6), confirming intracellular activation of Caco-2 MLCK.

Second, to confirm that Cyto B-induced increase in Caco-2 TJ permeability was mediated by MLCK activation, the effect of MLCK inhibitor 1-(5-iodonaphthalene-1-sulfonyl)-1H-hexahydro-1,4-diazepine (ML-7) on Cyto B modulation of Caco-2 TJ permeability was examined. The pretreatment of Caco-2 monolayers with ML-7, at the dose (15 μM) previously used by Turner et al. (42) to inhibit MLCK activity, prevented Cyto B activation of Caco-2 MLCK (Figs. 5 and 6). ML-7 (15 μM) also significantly prevented the Cyto B-induced drop in Caco-2 epithelial resistance and increase in paracellular permeability (Fig. 7). Similarly, other potent and selective MLCK inhibitors, including ML-9 and KT 5926 (37), also inhibited the Cyto B-induced drop in Caco-2 epithelial resistance (data not shown). These findings confirmed that Cyto B activation of Caco-2 MLCK was required for the Cyto B-induced increase in Caco-2 TJ permeability.

Third, to further validate the involvement of MLCK in this process, the effect of the specific myosin-Mg²⁺-ATPase inhibitor 2,3-butadione monoxime (BDM) on Cyto B-induced increase in Caco-2 TJ permeability was examined (17, 20). After MLCK catalyzed phosphorylation of MLC, myosin-Mg²⁺-ATPase hydrolyzes ATP to generate the energy needed for the actin-myosin contraction (1, 22, 23). The BDM (20 mM) treatment significantly prevented the Cyto B-induced drop in Caco-2 epithelial resistance (Fig. 8A), further supporting the involvement of MLCK and actin-myosin interaction in this process. Finally, the effects of the non-specific metabolic inhibitors 2,4-dinitrophenol and sodium azide (which prevent actin-myosin contraction by depleting metabolic energy) on Cyto B-induced increase in Caco-2 TJ permeability were examined. Sodium azide (30 mM) and 2,4-dinitrophenol (1 mM) also significantly prevented the Cyto B-induced drop in Caco-2 epithelial resistance (Fig. 8, B and C). In contrast, protein synthesis inhibitors, cycloheximide (70 μM) and actinomycin D (1 μg/ml) did not have significant effect on Cyto B-induced drop in Caco-2 epithelial resistance (data not shown).

Glucose involvement in Cyto B modulation of TJ barrier. Previous studies (42) have indicated that activation of glucose transport system results in an MLCK-mediated increase in TJ permeability. Because Cyto B is known to inhibit facilitative glucose transport (8, 9), in the following studies the effect of cytochalasin D (Cyto D) (which has no effect on glucose transport) on Caco-2 epithelial resistance was examined. Cyto D (10 μg/ml) caused a progressive drop in epithelial resistance (Fig. 9A). This was also associated with an increase in MLCK activity. Moreover, ML-7 and BDM

![Fig. 8. Effect of myosin-Mg²⁺-ATPase, metabolic energy, and protein synthesis inhibitors on Cyto B (5 μg/ml)-induced drop in Caco-2 epithelial resistance. The effect of 2,3-butadione monoxime (BDM; 20 mM; A); sodium azide (30 mM; B); and 2,4-dinitrophenol (1.0 mM; C) on Cyto B modulation of Caco-2 epithelial resistance expressed in Ω·cm². Values are means ± SE; n = 4.](http://ajpgi.physiology.org/)
also prevented the Cyto D-induced drop in Caco-2 epithelial resistance (data not shown).

Additionally, in separate studies, the Cyto B effect on Caco-2 epithelial resistance was examined in the absence of glucose in the incubation solution. Cyto B (10 μg/ml), in the absence of glucose in the incubation solution, produced a similar drop in Caco-2 epithelial resistance (Fig. 9B). As above, ML-7 and BDM also inhibited Cyto B-induced decrease in Caco-2 epithelial resistance (data not shown). These findings indicated that cytochalasin-induced alteration of Caco-2 TJ barrier function was not dependent on its modulation of glucose transport.

Role of MLCK in Cyto B modulation of Caco-2 actin filaments. As described above, Cyto B causes two distinct types of changes in Caco-2 actin filaments: early (≤1 min) fragmentation of actin filaments and late (15–30 min) actin clump formation (Fig. 1). In the following studies, the involvement of MLCK in the early and the late-phase changes in Caco-2 actin filaments was examined. The pretreatment of Caco-2 monolayers with ML-7 (MLCK inhibitor) did not affect early (<1 min) Cyto B severing or fragmentation of actins (Fig. 10, A–C). On the other hand, ML-7 (15 μM) prevented the late-phase (30 min) actin clump formation and enhanced actin fragment formation (Fig. 10, E and F), suggesting that MLCK activation is necessary for the conversion of actin fragments into actin clumps. Consistent with this, myosin-Mg²⁺-ATPase inhibitor (BDM) and metabolic inhibitors also did not affect early phase actin fragmentation, but prevented late-phase actin clump formation (Figs. 10, D and G, and 3, A and B).

Cyto B modulation of Caco-2 myosin filaments. Because MLCK activation triggers actin-myosin interaction, the effect of Cyto B-induced MLCK activation on myosin II filaments was examined by immunofluorescent antibody labeling. In the Caco-2 intestinal epithelial cells, myosin II filaments were localized in a belt-like manner near the apical perijunctional areas in the
region of zonula adherens, and mirrored actin microfilament distribution (Fig. 11A). Within 1 min of exposure to Cyto B (5 μg/ml), perijunctional myosin filaments became disassembled and displaced from the perijunctional regions, forming a discrete circular pattern near the cellular borders (Fig. 11B). On longer exposure, displaced myosin filaments reorganized into larger cytoskeletal clumps near the cellular periphery, similar to the actin filament distribution (Fig. 11). The pretreatment of Caco-2 monolayers with ML-7 prevented both early and late-phase changes in perijunctional myosin filaments (Fig. 12, A and B). Similarly, BDM (Fig. 12, C and D) and sodium azide (Fig. 12, E and F) also prevented Cyto B-induced alteration of myosin filaments, suggesting that MLCK activation and myosin-Mg\(^{2+}\)-ATPase activity were required for the Cyto B modulation of myosin filaments.

**Association between alteration of actin-cytoskeleton and ZO-1 TJ protein.** The increase in intestinal epithelial TJ permeability is associated with alteration of TJ structure (11, 26, 27). In the following studies, the structural correlation between Cyto B-induced alteration of actin-myosin cytoskeleton and TJ proteins was examined by immunofluorescent antibody labeling of the ZO-1 proteins. In the confluent Caco-2 monolayers, ZO-1 proteins were localized at the apical cellular borders and appeared as a continuous dense band (Fig. 13A). Cyto B (5 μg/ml) produced a marked disruption of the ZO-1 proteins with a breakage in the continuity of the ZO-1 band and separation of the ZO-1 proteins away from the cellular periphery (Fig. 13B). The Cyto B disruption and separation of the ZO-1 proteins from the cellular periphery visually correlated with the functional increase in Caco-2 TJ permeability. ML-7, BDM, and sodium azide prevented the Cyto B-induced disruption of the ZO-1 proteins (Figs. 13, C–E), suggesting that MLCK activation and actin-myosin interaction were required for the downstream modulation of TJ proteins. In contrast, protein synthesis inhibitors (cycloheximide and actinomycin D) did not affect the Cyto B modulation of ZO-1 proteins (Fig. 13F).

**DISCUSSION**

The cytochalasin disruption of actin microfilaments results in an increase in intestinal epithelial TJ per-
meability (29, 33). The major aim of this study was to delineate some of the intracellular mechanisms involved in cytochalasin-induced increase in intestinal epithelial TJ permeability and also to bridge some of the gaps in knowledge regarding this issue. Specifically, the role of MLCK and actin-myosin interaction on Cyto B-induced increase in intestinal epithelial TJ permeability was investigated.

Our results suggest that Cyto B-induced increase in Caco-2 TJ permeability is an MLCK-dependent process, requiring MLCK activation. Our studies indicate that inhibition of Cyto B-induced increase in Caco-2 MLCK activity prevents the increase in Caco-2 TJ permeability. Because actin-myosin interaction is dependent on both MLCK (induces MLC phosphorylation) and myosin-Mg\(^{2+}\)-ATPase (hydrolyzes ATP to generate energy needed for actin-myosin contraction) activation, the inhibition of Cyto B-induced increase in Caco-2 TJ permeability by myosin-Mg\(^{2+}\)-ATPase and metabolic inhibitors further supports the involvement of MLCK pathway in this process. In aggregate, our findings suggest that Cyto B-induced activation of Caco-2 MLCK triggers a sequence of intracellular processes including myosin-Mg\(^{2+}\)-ATPase activation and perijunctional actin-myosin interaction, which culminates in the functional opening of the Caco-2 TJ barrier.

The Cyto B stimulation of Caco-2 MLCK activity could have resulted from either an increase in MLCK expression or an increase in the activity of the preexisting MLCK proteins. Our findings that Cyto B does not affect Caco-2 MLCK protein level suggest that Cyto B-induced increase in MLCK activity was due to an increase in activity of preexisting MLCK protein and not increased expression of MLCK proteins. In this regard, protein synthesis inhibitors do not prevent Cyto B modulation of actin-myosin filaments or TJ permeability.

As to the mechanism of cytochalasin action on actin filaments, two separate processes have been previously described, an energy-independent and an energy-dependent process. Schliwa (39) demonstrated that cytochalasin exposure of African green monkey kidney cells (BS1 cells) produces an immediate severing or breakage of actin filaments into smaller fragments through an energy-independent process. Subsequently, severed actin fragments reorganize to form large cytoskeletal clumps consisting of actin, myosin, and tropomyosin through an energy-dependent process (39). Similarly, in this study, Cyto B also produced an energy-independent fragmentation of Caco-2 actin filaments within the first minute of Cyto B exposure. The metabolic inhibitors appeared to accentuate the formation of actin fragments (perhaps by inhibiting the energy-dependent processing of actin fragments). The late-phase actin clump formation was prevented by metabolic inhibitors, confirming the requirement of metabolic energy in the cytoskeletal clump formation.

Consistent with this, Madara et al. (33, 35) also reported that Cyto D exposure of the pig intestinal epithelium for 40 to 60 min produces a multifocal aggregation of cytoskeletal elements at various points along the perijunctional area with contraction of the enterocyte brush border and increase in TJ permeability. The Cyto D-induced aggregation of cytoskeletal elements and increase in TJ permeability were also prevented by the metabolic inhibitors (35).

Thus Cyto B disruption of Caco-2 actin appears to occur in 2 stages. First, Cyto B produces a direct fragmentation of actin filaments through an energy-independent process. Second, actin fragments are reorganized into large cytoskeletal clumps through an energy-dependent process. Our findings indicate that this energy-dependent conversion of actin fragments into large cytoskeletal clumps is prevented by MLCK and myosin-Mg\(^{2+}\)-ATPase inhibitors, suggesting that MLCK activation and subsequent myosin-Mg\(^{2+}\)-ATPase-induced actin-myosin interaction is required for this process. Because actin-myosin contraction is initiated by MLCK activation and myosin-Mg\(^{2+}\)-ATPase
activation (1, 23), our findings support a central role for actin-myosin contraction in the actin clump formation. Consistent with this, Colemen and Mooseker (6) previously demonstrated that villin-induced severing of actin filaments to smaller fragments also stimulates myosin-Mg\(^{2+}\)-ATPase activity.

Our results also indicate a sequential relationship between Cyto B disruption of actin filaments and alteration of myosin filaments. The Cyto B fragmentation of actins is associated with a rapid displacement of myosin filaments from the perijunctional regions. Within the first minute of Cyto B exposure, there is a rapid disassembly and displacement of myosin filament, forming a distinct circular pattern near the cellular borders. Subsequently, myosin filaments coalesce into large cytoskeletal clumps correlating with changes in actin filaments. In contrast to actins, both the early phase (<1 min) and the late-phase changes in the myosin filaments were inhibited by MLCK and myosin-Mg\(^{2+}\)-ATPase inhibitors, indicating that the early changes in myosin filaments were also dependent on MLCK activation. Because actin fragmentation results from a primary action of Cyto B and myosin alteration results as a secondary response to actin disruption, our findings suggest that actin fragmentation (the primary event) is responsible for the MLCK activation. The MLCK activation then presumably leads to the disassembly and displacement of the myosins (secondary response). In aggregate, these findings suggest that Cyto B-induced actin fragmentation produces Caco-2 MLCK activation, which in turn triggers actin-myosin interaction, leading to the displacement of the perijunctional myosin filaments from the cellular borders.

The Cyto B modulation of actin and myosin filaments was also associated with the morphological disruption of ZO-1 proteins, correlating with the functional increase in TJ permeability. The Cyto B disruption of ZO-1 proteins was prevented by MLCK, myosin-Mg\(^{2+}\)-ATPase and metabolic inhibitors, indicating that the downstream alteration of ZO-1 proteins is dependent on MLCK activation and actin-myosin interaction. These findings demonstrate a causal relationship between Cyto B activation of Caco-2 MLCK and subsequent modulation of the Caco-2 TJ proteins and the TJ barrier function.

As for the role of ZO-1 proteins in TJ barrier function, ZO-1 proteins have been previously proposed as a possible candidate protein linking TJs to the perijunctional cytoskeletal elements (2, 7, 10). In support of such a role, ZO-1 proteins have been shown to directly bind to actin filaments and to the transmembrane TJ protein occludin (10, 13, 21). ZO-1 proteins are a member of the membrane-associated guanylate kinase family (2, 41, 43). The members of this protein family are present on the cytoplasmic surface of specialized cell-to-cell contact and are involved in signal transduction and cytoskeletal organization (21, 43). Therefore, the proposed role of ZO-1 as an intermediary protein linking TJs to the cellular cytoskeleton is consistent with the known functions of this family of proteins (2, 7, 10). Our data, showing that Cyto B alteration of ZO-1 protein is linked to MLCK activation and actin-myosin interaction, support such a proposal.

In conclusion, our results provide new insight into the mechanism of Cyto B modulation of intestinal epithelial TJ barrier. Our results indicate that Cyto B-induced increase in Caco-2 intestinal epithelial TJ permeability is mediated by MLCK activation. It appears that Cyto B-induced MLCK activation triggers the perijunctional actin-myosin interaction leading to the downstream modulation of TJ proteins and barrier function.

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