Regulation of Tight Junction Permeability and Occludin Phosphorylation by RhoA-p160ROCK-dependent and independent Mechanisms*

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In epithelial and endothelial cells, tight junctions regulate the paracellular permeability of ions and proteins. Disruption of tight junctions by inflammation is often associated with tissue edema, but regulatory mechanisms are not fully understood. Using ECV304 cells as a model system, lysophosphatidic acid and histamine were found to increase the paracellular permeability of the tracer horseradish peroxidase. Cytoskeletal changes induced by these agents included stimulation of stress fiber formation and myosin light chain phosphorylation. Additionally, occludin, a tight junction protein, was a target for signaling events triggered by lysophosphatidic acid and histamine, events that resulted in its phosphorylation. A dominant-negative mutant of RhoA, RhoA T19N, or a specific inhibitor of Rho-activated kinases, Y-27632, prevented stress fiber formation, myosin light chain phosphorylation, occludin phosphorylation, and the increase in tracer flux in response to lysophosphatidic acid. In contrast, although RhoA T19N and Y-27632 blocked the cytoskeletal events induced by histamine, they had no effect on the stimulation of occludin phosphorylation or increased tracer flux, indicating that occludin phosphorylation may regulate tight junction permeability independently of cytoskeletal events. Thus, occludin is a target for receptor-initiated signaling events regulating its phosphorylation, and this phosphorylation may be a key regulator of tight junction permeability.

The tight junction (TJ) is localized to cell-cell contact sites in epithelial and endothelial cells. It serves as a paracellular barrier to restrict the movement of ions and proteins across tissue boundaries (1–4). This barrier function is essential for the maintenance of tissue environments. Dysfunction of the TJ occurs in response to a variety of inflammatory stimuli and also during ischemia, leading to tissue edema and damage. Therefore, analysis of TJ regulation could lead to an understanding of normal physiology as well as pathology and to the identification of novel therapeutic targets.

The molecular components of the TJ are being discovered and so far include ZO-1 (5), ZO-2 (6), ZO-3 (7), cingulin (8), 7H6 antigen (9), Rab3b (10), and symplekin (11). In addition to these peripheral membrane proteins, occludin was discovered as an integral membrane protein of the TJ having four transmembrane domains (12). The carboxy tail of occludin is linked to the actin cytoskeleton via ZO-1, ZO-2 and ZO-3 (13–15). Recent work has also identified members of the claudin family as TJ components that have four transmembrane domains but no sequence similarity to occludin (16–18). It has not yet been defined how these novel proteins interact with occludin or other TJ components. However, as the protein architecture of the TJ is revealed, analysis of function of the TJ on a molecular basis becomes possible.

The formation and maintenance of the TJ has been considered to be regulated not only by the specific proteins of cell-cell junctions but also by the perijunctional actin cytoskeleton (19). Botulinum C3 toxin, which ADP-ribosylates and inactivates Rho, has been shown to disrupt perijunctional actin, resulting in TJ dysfunction in epithelial cells (20). Also, mutants of RhoA and Rac1 disrupted TJ functions (21). Thus, signaling pathways transduced by the Ras-related small GTPase Rho family members like Rho and Rac1, which control the actin cytoskeleton, have been implicated in the regulation of the TJ (see Refs. 19 and 22), whereas involvement of downstream signaling events of Rho in TJ function is still lacking. Recently, several downstream targets of Rho have been identified (see Refs. 22 and 23). Among these, p160ROCK/Rho kinase (24, 25), one of the key effectors of Rho, has been shown to be a serine-threonine kinase that is involved in the regulation of actin organization, cellular morphology, and cellular transformation (26, 27).

In this study, the basis of effects of inflammatory stimuli on TJ permeability was investigated. The role of the cytoskeleton and the possibility of direct effects on occludin were explored. Using ECV304 cells as a model system, lysophosphatidic acid (LPA) and histamine were found to increase the paracellular permeability of the tracer horseradish peroxidase (HRP). LPA is a glyceroalkylphospholipid that is secreted from activated platelets, mediating tissue regeneration and wound healing (28), and can induce an increase in TJ permeability in brain endothelial cells (29). Also, histamine causes vascular leakage in vivo, but mechanisms are not completely clear yet (see Ref. 30).

Because LPA activates Rho and its targets (22, 28), the involve-
ment of these was studied using a dominant-negative mutant of RhoA (RhoA T19N) considered to be the inactive GDP-bound form of RhoA (31, 32) and a specific p160ROCK inhibitor (Y-27632 (25)). Evidence is provided that TJ permeability is regulated by RhoA-p160ROCK-dependent and -independent mechanisms and that occludin is a target for receptor-initiated signaling events regulating its phosphorylation.

**EXPERIMENTAL PROCEDURES**

**Reagents**—All tissue culture materials were from Life Technologies. In Cells Electrophoretic reagents were from Bio-Rad (Richmond, CA). [32P]orthophosphate (ICN Biomedicals, Costa Mesa, CA) for 4 h under conditions described previously (40).

**Replication-Defective Recombinant Adenovirus—**RhoA T19N, from Professor Yoshimi Takai (Osaka University, Osaka, Japan), or LacZ was purchased from Upstate Biotechnology (Lake Placid, NY). The anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibody against occludin was from Santa Cruz Biotechnology, Inc. (Arlington Heights, IL). Phospho-associated protein 2 (Pap2) was purchased from Cell Signaling Technology (Beverly, MA). Antibodies to ZO-1, occludin, and Ser19-phosphorylated myosin light chain (MLC) was raised against synthetic peptide RFQRAPLSVNFAMK (where p indicates phosphorylation), as described previously (35).

**Antibodies**—The rat monoclonal antibody (MO3G7) and rabbit polyclonal antibody were both raised against a fragment of occludin fused to glutathione S-transferase (34). The anti-ZO-1 monoclonal antibody and the anti-phosphotyrosine antibody PY20 were from Transduction Laboratories (Lexington, KY). The anti-phosphotyrosine antibody 4G10 was purchased from Upstate Biotechnology (Lake Placid, NY). The monoclonal antibody against occludin was from Santa Cruz Biotechnology, Inc. (Arlington Heights, IL). Phospho-associated protein 2 (Pap2) was purchased from Cell Signaling Technology (Beverly, MA). Antibodies to ZO-1, occludin, and Ser19-phosphorylated myosin light chain (MLC) was raised against the synthetic peptide RFQRAPLSVNFAMK (where p indicates phosphorylation), as described previously (35).

**Cell Culture—**ECV304 cells were obtained from the European Collection of Animal Cell Cultures (Salisbury, UK) and cultured at 37 °C under an atmosphere of 5% CO2 in DME containing 10% fetal calf serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Confluent cultures of ECV304 cells containing 100 units/ml penicillin, and 100 μg/ml streptomycin.

**Replication-Defective Recombinant Adenovirus—**RhoA T19N, from Professor Yoshimi Takai (Osaka University, Osaka, Japan), or LacZ was placed into pAdex1CAwt under a CA promoter comprising a cytomegalovirus promoter (40, 41).

**Recombinant Adenoviral Gene Transfer—**After ECV304 cells had been transfected with RhoA or LacZ and incubated for 24 h, the viral suspension was removed by washing twice with serum-depleted DME, and the cells were cultured with serum-depleted DME for 48 h.

**HRP Flux Measurement—**ECV304 cells were seeded onto 0.4-μm polycarbonate Transwell filters (Costar Corp., Cambridge, MA). After attaining confluence, the cells were incubated in serum-depleted DME with or without recombinant adenosine gene transfer for 48 h. For a particular compound, the final concentration of 10 μM was added, and the incubation was continued for 60 min. Medium was then replaced with fresh serum-free DME in the presence or absence of 10 μM Y-27632 and agonists at the indicated concentrations. To the upper chambers, HRP dissolved in serum-free DME was added to give a final concentration of 0.5 μM. The upper chambers contained 200 μl of medium, and the lower chambers contained 800 μl of medium. One hour after the start of the experiment, 50 μl of medium was collected from the lower chambers. The HRP content of the samples was evaluated spectrophotometrically by assaying peroxidase activity in buffer containing 0.5 mM guaiacol, 50 mM Na2HPO4, and 0.6 mM H2O2 and measuring absorbance at 470 nm. Data from five cells were then detected by autoradiography, and occludin protein labeled bands according to the procedures described previously (40).

**Immunofluorescence—**All procedures were performed at room temperature. Cells were fixed in 3% paraformaldehyde in PBS for 15 min. After fixation, the cells were rinsed and permeabilized by incubation with 0.2% Triton X-100 in PBS for 15 min. After rinsing, the cells were blocked in 1% BSA in PBS for 15 min and incubated with 100 μg/ml fluorescein isothiocyanate-conjugated phalloidin (Molecular Probes, Inc., Eugene, OR) in blocking solution for 60 min. After the final rinse, the cells were mounted with fluorescent mounting medium (DAKO, Carpinteria, CA) and examined using a Axioskop™ fluorescence microscope (Carl Zeiss, Inc., Thornwood, NY) fitted with 100× objectives. Photographs were taken using 400 ASA T-MAX film (Eastman Kodak Co.).
After transfer to nitrocellulose, [32P]phosphate incorporated into protein was detected by autoradiography, and signal corresponding to occludin protein was then revealed by subsequent immunoblotting of the filter with anti-occludin antibody.

Phosphatase treatment of radiolabeled occludin was performed essentially as described by Meisenhelder and Hunter (41). Thus, an immune complex containing occludin from [32P]orthophosphate-labeled cells was prepared as described above. To remove interfering salts and detergents, the beads (50-μl packed volume) were washed twice with 1 ml of wash buffer containing 1% Triton X-100, 0.1 M NaCl, 25 mM Hepes-NaOH, pH 7.4. They were then switched to phosphatase buffer by washing twice with a solution containing 20 mM Mops-NaOH, 1 mM MgCl₂, 0.8 mM diithiothreitol, 4 μg/ml leupeptin, 4 μg/ml soybean trypsin inhibitor, pH 5.5. The beads were then incubated with 50 μl of the phosphatase buffer in the absence or presence of 0.2 units of potato acid phosphatase (Calbiochem). Phosphatase activity was blocked as described (41). After 1 h at 37 °C, the reaction was quenched by the addition of 1 ml of ice-cold wash buffer. The suspension was briefly (<10 s) centrifuged to pellet the beads. Protein was eluted into Laemmli sample buffer, resolved by SDS-PAGE, and transferred to nitrocellulose as described above. [32P]Phosphate incorporated into protein was detected by autoradiography, and occludin protein was revealed by immunoblotting.

RESULTS

LPA and Histamine Increase Paracellular Flux of HRP in ECV304 Cells—The effects of LPA on the TJ permeability of ECV304 cell monolayers were investigated by measuring paracellular flux of HRP. Using cultures on Transwell filters, HRP was added to the apical chambers in the presence or absence of LPA. HRP that passed via the paracellular pathway to enter the basolateral chamber was quantified by assaying peroxidase activity spectrophotometrically. The HRP activity detected was compared with that of control cells. As shown in Fig. 1a, LPA induced a greater flux of HRP in a dose-dependent manner. The involvement of RhoA was examined by using adenovirus-mediated overexpression of RhoA T19N, a dominant-negative mutant of RhoA. LacZ overexpression, confirmed by X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside) staining (data not shown), was used as a control. In these experiments, the increase in HRP flux in response to LPA was similar in noninfected cells and LacZ-overexpressing cells. In contrast, LPA failed to stimulate an increase in HRP flux in RhoA T19N-overexpressing cells (Fig. 1b). Next, the role of p160ROCK was investigated using the specific p160ROCK inhibitor Y-27632 (25). Pretreatment with 10 μM Y-27632 blocked the increase in HRP flux induced by LPA (Fig. 1b). These data indicate that RhoA and its target p160ROCK transduce the action of LPA to increase TJ permeability in ECV304 cells.

The effects of histamine on HRP flux in ECV304 cells were then examined. Like LPA, histamine increased HRP flux in a dose-dependent manner in ECV304 cells (Fig. 2a). The roles of RhoA and p160ROCK were again studied. In noninfected cells and LacZ-overexpressing cells, histamine increased HRP flux to similar levels (Fig. 2b). However, overexpression of RhoA T19N had no significant effect on the increase in HRP flux induced by histamine (Fig. 2b). Also, inhibition of p160ROCK using Y-27632 failed to inhibit the increase in HRP flux in response to histamine (Fig. 2b). Thus, in contrast to the response to LPA, histamine appears to stimulate an increase in TJ permeability in ECV304 cells independently of RhoA and p160ROCK.

Changes in the Actin Cytoskeleton in Response to LPA and Histamine—To determine whether LPA and histamine caused changes in the actin cytoskeleton in ECV304 cells, F-actin was visualized by staining with fluorescein isothiocyanate-conjugated phalloidin (Fig. 3). In noninfected cells and LacZ-overexpressing cells as controls, pericellular actin bundles were seen, and stress fibers were hardly detectable in the cell bodies (Fig. 3, a and b), whereas subtle reorganization of pericellular actin bundles was observed in RhoA T19N-overexpressing cells and cells pretreated with 10 μM Y-27632 (Fig. 3, c and d). Noninfected cells and LacZ-overexpressing cells that were treated with 1 μM LPA showed F-actin bundles in stress fibers and some gaps between cells (Fig. 3, e and f). In contrast, in the cells overexpressing RhoA T19N, LPA failed to induce F-actin bundles (Fig. 3g). Pretreatment with 10 μM Y-27632 also prevented stress fiber formation in response to LPA (Fig. 3h).

Similar to the effect of LPA, 1 μM histamine induced F-actin bundles in stress fibers in noninfected cells and LacZ-overexpressing cells (Fig. 3, i and j). Both overexpression of RhoA T19N and pretreatment with Y-27632 also blocked formation of stress fibers in response to histamine (Fig. 3, k and l). These data show that LPA and histamine induce reorganization of pericellular actin bundles and stimulation of stress fibre formation in ECV304 cells and that both events are mediated by RhoA and p160ROCK.

Changes in Occludin Electrophoretic Mobility in Response to LPA and Histamine—Although LPA and histamine clearly had effects on the actin-based cytoskeleton, the possibility of direct effects on TJ proteins was also explored. The main reason for this was because the cytoskeletal effects of histamine were blocked by inhibition of RhoA-p160ROCK signaling, whereas effects on TJ permeability were unaffected. By immunocytochemistry, it was shown that the localization of occludin or ZO-1 was not altered in response to either LPA or histamine (data not shown). The possibility of effects of LPA and histamine on biochemical changes in the TJ protein occludin was
Both immunological and labeling procedures were used. Occluded to see if these changed in response to cell stimulation. Phosphorylated in occludin were then characterized and investigated. Metabolically labeled with [32P]orthophosphate, and phosphorylation of occludin was detected. However, after precipitation and resolution by SDS-PAGE. In control cells, phosphorylation of occludin was not detected with PY20 or 4G10 (Fig. 5b). As a control, cells were treated with pervanadate, a membrane-permeable peroxide derivative of vanadate and a potent inhibitor of tyrosine phosphatases (see Ref. 33). Occludin from pervanadate-treated cells showed a change in electrophoretic mobility and clear immunoreactivity with PY20 and 4G10 (Fig. 5b). Phosphorylation was then analyzed by phosphoamino acid analysis of metabolically radiolabeled occludin using high voltage electrophoresis in two dimensions. Occludin from control cells was phosphorylated mainly on serine residues, phosphothreonine was barely detectable, and phosphotyrosine was not detected (Fig. 5c). The phosphoamino acid composition of occludin from LPA and histamine-treated cells was very similar to that of control cells (Fig. 5c). Thus, phosphorylation of occludin may involve subtle changes in phosphorylation of serine or threonine residues. Tyrosine phosphorylation does not seem to play a role.

Two-dimensional Gel Analysis—Occludin phosphorylation in [32P]orthophosphate-labeled cells was also analyzed by two-dimensional gel electrophoresis. Occludin from control cells migrated as a series of at least six discrete spots (Fig. 6a, panel a, arrows), suggesting differentially, post-translationally modified protein. The five most acidic spots appeared as doublets consisting of a more abundant lower spot and a less abundant, slightly retarded, more acidic spot. Out of the six spots, the most basic was not detectable as phosphorylated (Fig. 6a, cf. panels a and b, arrows). As the pl of occludin decreased, phospho-phosphate was detected mainly in the lower, more abundant component of the pairs. After stimulation with histamine (Fig. 6a, panels c and d) or LPA (Fig. 6a, panels e and f), occludin still migrated as a series of spots. However, the migration of these spots was altered (Fig. 6a, panels c through f, arrows). The biochemical basis of the change in electrophoretic mobility of occludin was investigated. One possibility was that this was due to changes in phosphorylation of the protein. In initial experiments, cells were metabolically labeled with [32P]orthophosphate, and phosphorylation of occludin was examined after immunoprecipitation and resolution by SDS-PAGE. In control cells, phosphate incorporation into occludin was detected. However, after stimulation with histamine or LPA, even though a band shift in occludin was observed, an increase in phosphate labeling of the protein was not detected (Fig. 5a). The intensity of labeled bands was quantitated by densitometry, and similar results were obtained in other experiments.

Phosphoamino Acid Analysis—Amino acid residues phosphorylated in occludin were then characterized and investigated to see if these changed in response to cell stimulation. Both immunological and labeling procedures were used. Occludin immunoprecipitates from cells treated with either 1 [mu]M LPA or 1 [mu]M histamine were resolved by SDS-PAGE and immunoblotted with anti-occludin antibody, revealing the mobility shift, and then with the anti-phosphotyrosine antibodies PY20 or 4G10 (Fig. 5b). As a control, cells were treated with pervanadate, a membrane-permeable peroxide derivative of vanadate and a potent inhibitor of tyrosine phosphatases (see Ref. 33). Occludin from pervanadate-treated cells showed a change in electrophoretic mobility and clear immunoreactivity with PY20 and 4G10 (Fig. 5b). In contrast, tyrosine phosphorylation of occludin was not detected with PY20 or 4G10 in immunoprecipitates from cells stimulated with LPA or histamine (Fig. 5b).

Phosphorylation was then analyzed by phosphoamino acid analysis of metabolically radiolabeled occludin using high voltage electrophoresis in two dimensions. Occludin from control cells was phosphorylated mainly on serine residues, phosphothreonine was barely detectable, and phosphotyrosine was not detected (Fig. 5c). The phosphoamino acid composition of occludin from LPA and histamine-treated cells was very similar to that of control cells (Fig. 5c). Thus, phosphorylation of occludin may involve subtle changes in phosphorylation of serine or threonine residues. Tyrosine phosphorylation does not seem to play a role.
was different from that of occludin from vehicle-treated cells. In particular, the distribution of occludin between the two spots in the more acidic forms of the protein was shifted such that the upper member of the pair was predominant or more equal to that of the lower member of the pair. This is seen fairly well with the second least basic form of occludin (Fig. 6A, panels c–f, arrows). Enlarged views of these regions of the blots are shown in Fig. 6B. Although a detectable increase in phosphorylation of occludin was not observed, the decrease in pI of the protein is consistent with increased phosphorylation. The lack of increase in detectable phosphorylation may again be due to the fact that occludin in resting cells is already substantially phosphorylated, and the phosphorylation responsible for the pI and band shift is difficult to detect in this background. Nevertheless, the two-dimensional gel analysis is consistent with the possibility that occludin phosphorylation is altered in response to histamine and LPA treatment.

Effects of in Vitro Phosphatase Treatment—Another approach to investigate if phosphorylation is responsible for electrophoretic mobility changes in a protein is to study the effects of in vitro phosphatase treatment (see Ref. 41). Again, cells were labeled with [32P]orthophosphate and then treated for 10 min with vehicle (Cont.), 1 μM histamine (Hist.) or 1 μM LPA. Occludin was extracted, immunoprecipitated, resolved by SDS-PAGE, and detected by immunoblotting (Protein) or autoradiography (32P). Occludin from control cells migrates as a single band but becomes a doublet after treatment of cells with LPA or histamine (arrowheads). However, as confirmed by densitometry, changes in total phosphorylation of occludin were not detectable. Panel b, occludin immunoprecipitates (IP) from cells stimulated for 10 min with either vehicle (Cont.), 1 μM LPA, 1 μM histamine (Hist.), or 100 μM pervanadate (PV) were resolved by SDS-PAGE and immunoblotted with either anti-occludin antibody or the anti-phosphotyrosine antibodies PY20 and 4G10. The brackets indicate more slowly migrating bands of occludin from cells treated with pervanadate. Panel c, the bands corresponding to occludin in the autoradiogram of panel a were excised and subject to partial acid hydrolysis, and the released phosphoamino acids were analyzed by high voltage electrophoresis in two dimensions. The migration of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y), as detected by ninhydrin staining, is indicated in the Standards panel.

Fig. 4. Occludin electrophoretic mobility was altered in response to LPA and histamine. Occludin was immunoprecipitated with an anti-occludin polyclonal antibody from cells stimulated with LPA (a) and histamine (b) at various doses (for 10 min) and times (at 1 μM) as indicated. Immunoprecipitates were resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-occludin monoclonal antibody (MOC37). As a control, 40 μg of protein of whole cell lysates from cells in the same condition were resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-ZO-1 antibody (a and b).

Fig. 5. Phosphorylation analysis of occludin. Panel a, cells were metabolically labeled with [32P]orthophosphate and then treated for 10 min with vehicle (Cont.), 1 μM histamine (Hist.) or 1 μM LPA. Occludin was extracted, immunoprecipitated, resolved by SDS-PAGE, and detected by immunoblotting (Protein) or autoradiography (32P). Occludin from control cells migrates as a single band but becomes a doublet after treatment of cells with LPA or histamine (arrowheads). However, as confirmed by densitometry, changes in total phosphorylation of occludin were not detectable. Panel b, occludin immunoprecipitates (IP) from cells stimulated for 10 min with either vehicle (Cont.), 1 μM LPA, 1 μM histamine (Hist.), or 100 μM pervanadate (PV) were resolved by SDS-PAGE and immunoblotted with either anti-occludin antibody or the anti-phosphotyrosine antibodies PY20 and 4G10. The brackets indicate more slowly migrating bands of occludin from cells treated with pervanadate. Panel c, the bands corresponding to occludin in the autoradiogram of panel a were excised and subject to partial acid hydrolysis, and the released phosphoamino acids were analyzed by high voltage electrophoresis in two dimensions. The migration of phosphoserine (S), phosphothreonine (T) and phosphotyrosine (Y), as detected by ninhydrin staining, is indicated in the Standards panel.
phosphatase (recombinant protein produced in *Escherichia coli*), in a dose-dependent manner, could reverse the effects of both LPA and histamine on the occludin band shift (Fig. 7b). Furthermore, the activities of both alkaline phosphatase and λ phosphatase were blocked by phosphatase inhibitors (Fig. 7c).

The ability of phosphatases to reverse the effects of LPA and histamine on the occludin band shift *in vitro* suggests that the band shift is due to occludin phosphorylation. Because occludin is already phosphorylated in control cells, LPA and histamine probably produce subtle but site-specific changes in occludin phosphorylation. Such changes would be sufficient to result in altered electrophoretic mobility, both in the form of an acid shift (see Fig. 6) and apparent size shift of the protein but not enough to detect changes in total phosphorylation of occludin.

**Involvement of RhoA and p160ROCK in Occludin Phosphorylation**—The involvement of RhoA and p160ROCK in occludin phosphorylation induced by LPA and histamine was investigated. In noninfected cells and cells overexpressing LacZ as a negative control, LPA and histamine again induced an upward band shift of occludin (Fig. 8). Overexpression of RhoA T19N prevented the LPA-induced occludin band shift but not that in response to histamine (Fig. 8, a and b). The expression of RhoA was determined by the immunoblotting of whole cell lysates from each condition (Fig. 8, a and b). RhoA expression was similar in noninfected cells, LacZ-overexpressing cells, and cells pretreated with Y-27632. The anti-RhoA antibody detected overexpressed RhoA T19N (Fig. 8, a and b). Also, pretreatment with Y-27632 blocked the ability of LPA to cause an occludin band shift (Fig. 8a). In contrast, the histamine-induced band shift of occludin was not blocked by Y-27632 (Fig. 8b). As a control, expression levels and electrophoretic mobility of ZO-1 were unaffected (Fig. 8, a and b). These data suggest that RhoA and p160ROCK mediate LPA-induced occludin phosphorylation. In contrast, histamine-stimulated occludin phosphorylation, like its effect on tight junction permeability (Fig. 2), appears to be mediated by a pathway that is independent of RhoA and p160ROCK.

**MLC Phosphorylation in Response to LPA and Histamine**—MLC phosphorylation mediated by Rho and its target Rho kinase/p160ROCK has been suggested to be a key regulator of cell retraction leading to increased TJ permeability in endothelial cells (42, 43). Therefore, the effects of LPA and histamine on MLC phosphorylation in ECV304 cells were examined. After stimulation with 1 μM LPA or 1 μM histamine, equal protein amounts of cell lysates were resolved by SDS-PAGE and then analyzed by immunoblotting (Fig. 8, a and b) with an antibody that recognizes Thr18- and Ser19-phosphorylated MLC (PP-MLC). Some of the immunoprecipitates (left panels) were subject to a sham treatment with potato acid phosphatase (Pase) or λ protein phosphatase (LP). Phosphatase treatment dose-dependently reversed the band shift. Panel c, the activity of phosphatase seen in panel b was not observed in the presence of a mixture of phosphatase inhibitors (PI).
in cell lysates. In contrast, LPA and histamine failed to increase PP-MLC immunoreactivity in RhoA T19N-overexpressing cells and cells pretreated with Y-27632. These results indicate that LPA and histamine induce MLC phosphorylation via RhoA and p160ROCK in ECV304 cells.

**Disrupting TJs Does Not Stimulate Occludin Phosphorylation**—It is possible that an increase in occludin phosphorylation is responsible for increased TJ permeability. Alternatively, increased TJ permeability may trigger occludin phosphorylation. To address this issue, an actin-depolymerizing agent, cytochalasin D (45), was used to disrupt TJs, and effects on occludin phosphorylation were examined. The efficacy of cytochalasin D on TJ structures was confirmed by staining cells with the extracellular calcium chelating agent BAPTA (4 mM, 10 μM Y-27632 for 60 min that were stimulated for 10 min with either 1 μM LPA (a) or 1 μM histamine (b)). Immunoprecipitates were resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-occludin antibody. Whole cell lysates (40 μg of protein) from each condition were also resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-ZO-1 antibody, an anti-RhoA antibody, and an anti-PP-MLC antibody that recognizes MLC phosphorylated on Thr18 and Ser19 residues.

**Fig. 8. Involvement of RhoA and p160ROCK in occludin phosphorylation and MLC phosphorylation induced by LPA and histamine.** Occludin was immunoprecipitated from noninfected cells, LacZ-overexpressing cells, RhoA T19N-overexpressing cells, or cells pretreated with 10 μM Y-27632 for 60 min that were stimulated for 10 min with either 1 μM LPA (a) or 1 μM histamine (b). Immunoprecipitates were resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-occludin antibody. Whole cell lysates (40 μg of protein) from each condition were also resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-ZO-1 antibody, an anti-RhoA antibody, and an anti-PP-MLC antibody that recognizes MLC phosphorylated on Thr18 and Ser19 residues.

**Fig. 9. Disruption of TJs does not cause occludin phosphorylation.** Panel a, after treatment of ECV304 cells with either vehicle or 1 μM cytochalasin D for 30 min, cells were fixed, and immunolabeling of occludin was performed. Occludin was localized at cell-cell contacts in vehicle-treated cells (left). In contrast, in cytochalasin D-treated cells (right), occludin localization to the cellular TJs was disrupted. Bar, 10 μm. Panel b, cells were stimulated for 10 min with either 1 μM LPA or 1 μM histamine after pretreatment with either vehicle or 1 μM cytochalasin D for 30 min. Occludin immunoprecipitates and whole cell lysates (40 μg protein) from each condition were resolved by SDS-PAGE and then analyzed by immunoblotting with an anti-occludin or, as a control, an anti-ZO-1 antibody, respectively.

**DISCUSSION**

The present study shows that RhoA and p160ROCK are components of a signaling pathway coupling LPA receptor stimulation to changes in TJ permeability. However, other pathways must exist because the physiologically similar effect of histamine was independent of RhoA-p160ROCK. Furthermore, the TJ protein occludin is shown to be the target for G protein-coupled receptor-initiated signaling pathways. Again, a pathway involving RhoA-p160ROCK is shown to exist, but RhoA-p160ROCK-independent signaling to occludin can also occur.

Regulation of the function of TJs is considered to be achieved in a concerted manner by both the cytoskeleton and specific junctional proteins (1, 2, 19, 46). Regarding the cytoskeleton, inactivation of the Ras-related GTPase Rho by botulinum C3 toxin was shown to lead to perijunctional actin reorganization and barrier dysfunction of TJ (20), suggesting that perijunctional cytoskeleton regulated by Rho can influence TJ permeability (19). Also, cell contraction mediated by MLC phosphorylation may play an important role in TJ permeability control (44). The pathways activating MLC phosphorylation appear to be transduced by intracellular Ca2+ as well as Rho-associated kinase/p160ROCK, a downstream target of Rho (44, 47).

Here, using ECV304 cells as a model system, LPA and histamine were shown to have similar abilities to increase TJ permeability. Both agents also caused not only actin reorganization, such as the formation of F-actin bundles and disappearance of perijunctional actin (Fig. 3), but also MLC phosphorylation (Fig. 8). Blocking either RhoA signaling (by
overexpression of the dominant negative RhoA T19N or the activity of the RhoA effector p160ROCK (using the pharmacological inhibitor Y-27632) prevented these cytoskeletal changes in response to LPA and histamine (Figs. 3 and 8). However, although RhoA T19N or Y-27632 blocked the LPA-induced increase in TJ permeability, they had no effect on the response to histamine (Figs. 1 and 2). Therefore, in the case of LPA, RhoA and p160ROCK are critically involved in the stimulated increase in TJ permeability. In contrast, histamine has effects on TJ permeability that are independent of both RhoA-p160ROCK and effects on the cytoskeleton. Therefore, other mechanistic possibilities were explored.

Direct effects of signaling on protein components of the TJ were examined. By immunoblot analysis, stimulation of cells with either LPA or histamine obviously had an effect on occludin, causing its electrophoretic retardation when analyzed by SDS-PAGE. The dose dependence of this effect was similar to that required for the increases in TJ permeability. The biochemical basis of this effect on occludin was analyzed and was highly likely due to an alteration in phosphorylation. Indeed, the reversal of the LPA and histamine-induced band shift in occludin by in vitro phosphatase treatment is consistent with the band shift due to an increased phosphorylation. The anti-phosphotyrosine antibodies PY20 and 4G10 did not react with occludin from stimulated cells, suggesting that the decrease in electrophoretic mobility of occludin was not due to an increase in its tyrosine phosphorylation. By metabolic labeling with \(^{32}\)P phosphate, occludin was shown to be phosphorylated mainly on serine residues in control cells. However, even after stimulation with LPA or histamine, it was difficult to detect an increase in phosphorylation of either serine or threonine residues. Presumably, changes in phosphorylation of occludin are difficult to detect because of the high basal level of phosphorylation. The changes are inferred to be on serine or threonine residues because of our inability to detect tyrosine phosphorylation either by immunoblotting or phosphoamino acid analysis from labeled cells. The complexity of occludin phosphorylation was revealed by two-dimensional gel analysis. Ocludin from control cells migrated as a series of pI variants, the more acidic of which were detectable as phosphorylated. LPA or histamine stimulation again resulted in changes in electrophoretic mobility of occludin, notably to more acidic, electrophoretically retarded forms, consistent with increased phosphorylation. Even by two-dimensional gel analysis it was difficult to detect gross changes in occludin phosphorylation, but again, this appears to be due to the high basal level of phosphorylation.

Phosphorylation of occludin has now been reported in several different situations. Sakakibara et al. (48) show that occludin is phosphorylated on serine-threonine residues during the formation of cell-cell contacts in MDCK cells. During the TJ assembly of Xenopus laevis embryos, occludin dephosphorylation was observed (49). Also vascular endothelial growth factor stimulated TJ permeability and occludin phosphorylation in retinal endothelial cells (50). Regarding sites of phosphorylation and function, the relationship between occludin phosphorylation in these situations is not clear.

It is possible that occludin phosphorylation may be a cause or consequence of increased TJ permeability. In our study, we would suggest that it may be causal and, also, capable of being mechanistically independent of changes in the cytoskeleton. In cells where TJ structure was disrupted by treatment with cytochalasin D or BAPTA, occludin phosphorylation was not affected. In these same cells, effects of LPA and histamine could still be observed. Thus, occludin phosphorylation (the band shift) does not appear to be a consequence of disruption of junctions, suggesting that it may play a causal role. In the case of LPA, a RhoA-p160ROCK pathway couples receptor-stimulated signaling events to changes in the actin cytoskeleton (stress fiber formation, MLC phosphorylation), changes in TJ permeability, and occludin phosphorylation. With LPA, because all events were blocked by interfering with the RhoA-p160ROCK pathway, it is difficult to ascribe functional importance to any of them in particular. However, with histamine, a different situation was found. In this case, blocking the RhoA-p160ROCK pathway had differential effects on the cytoskeleton, occludin phosphorylation, and increase in TJ permeability. Interfering with RhoA-p160ROCK signaling blocked the cytoskeletal changes in response to histamine, whereas both occludin phosphorylation and the increase in TJ permeability were unaffected. This raises the possibility that occludin phosphorylation may be a key regulator of TJ permeability, acting independently of cytoskeletal events.

The precise mechanisms involved in occludin phosphorylation and changes in TJ permeability have yet to be elucidated. It is not clear whether LPA and histamine use parallel, independent signaling pathways to regulate occludin phosphorylation. p160ROCK appears to be a component of the signaling pathway activated by LPA but not by histamine. Another possibility is that different pathways are activated with convergence, perhaps at the point of a common kinase responsible for occludin phosphorylation. How phosphorylation changes in occludin may regulate TJ permeability has yet to be understood. Conformational changes of occludin might affect the association with peripheral membrane proteins of the TJ linked to the actin cytoskeleton. However, in our present study, LPA and histamine did not have any apparent effects on the localization of TJ proteins occludin and ZO-1 (data not shown), indicating that this aspect of the protein architecture of the TJ is maintained after stimulation with these agonists. Also, the interaction of occludin with other TJ membrane proteins such as claudins (16–18) might alter in response to phosphorylation.

In conclusion, our results demonstrate that TJ permeability is regulated by distinct mechanisms that are RhoA-p160ROCK-dependent and -independent. RhoA and p160ROCK appear to be crucial regulators of the cytoskeleton that may partly determine TJ permeability. Also, evidence is provided that receptor-initiated signaling events can trigger phosphorylation of the TJ-specific membrane protein occludin on serine-threonine residues via RhoA-p160ROCK-dependent and RhoA-p160ROCK-independent pathways. Recently, LPA was found to be accumulated in human atherosclerotic lesions that are prone to thrombotic complications, indicating LPA as an atherothrombogenic molecule (51). The analysis of regulatory mechanisms controlling TJ permeability could lead to the understanding of the physiology as well as the pathology of vascular disorders caused by inflammation, ischemia, and atherosclerosis and, therefore, direct us to the identification of novel therapeutic targets.

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