Protein Kinase Cα-RhoA Cross-talk in CCL2-induced Alterations in Brain Endothelial Permeability*

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Monocyte chemoattractant protein-1 (MCP-1 or CCL2) regulates blood-brain barrier permeability by inducing morphological and biochemical alterations in the tight junction (TJ) complex between brain endothelial cells. The present study used cultured brain endothelial cells to examine the signaling networks involved in the redistribution of TJ proteins (occludin, ZO-1, ZO-2, claudin-5) by CCL2. The CCL2-induced alterations in the brain endothelial barrier were associated with de novo Ser/Thr phosphorylation of occludin, ZO-1, ZO-2, and claudin-5. The phosphorylated TJ proteins were redistributed/localized in Triton X-100-soluble as well as Triton X-100-insoluble cell fractions. Two protein kinase C (PKC) isoforms, PKCα and PKCζ, had a significant impact on this event. Inhibition of their activity using dominant negative mutants PKCα-DN and PKCζ-DN diminished CCL2 effects on brain endothelial permeability. Previous data indicate that Rho/Rho kinase signaling is involved in CCL2 regulation of brain endothelial permeability. The interactions between the PKC and Rho/Rho kinase pathways were therefore examined. Rho, PKCα, and PKCζ activities were knocked down using dominant negative mutants (T17Rho, PKCα-DN, and PKCζ-DN, respectively). PKCα and Rho, but not PKCζ and Rho, interacted at the level of Rho, with PKCα being a downstream target for Rho. Double transfection experiments using dominant negative mutants confirmed that this interaction is critical for CCL2-induced redistribution of TJ proteins. Collectively these data suggest for the first time that CCL2 induces brain endothelial hyperpermeability via Rho/PKCα signal pathway interactions.

Endothelial paracellular permeability is tightly regulated by a dynamic equilibrium between the contractile forces generated by the endothelial cell cytoskeleton and adhesion forces generated by endothelial cell-cell and cell-matrix adhesion sites (1, 2). An equilibrium shift between these forces leads to alterations in paracellular permeability. For example, proinflammatory stimuli such as histamine, bradykinin, platelet-activating factor, growth factors, cytokines, and reactive oxygen species cause increased paracellular permeability by inducing endothelial cell retraction (2–4). The morphological changes associated with endothelial cell contraction include alterations in the cytoskeleton (reorganization of F-actin microfilaments, loss of peripheral actin band, and marked increases in F-actin stress fiber formation) and the tight and adherens junction structures (disassembly and/or redistribution of junction proteins) (1, 2).

The molecular mechanisms underlying regulation of endothelial permeability are still unresolved. One line of evidence highlights the importance of endothelial cell contractility in controlling microvascular permeability, revealing that contraction of endothelial cells is dependent on the phosphorylation state of the regulatory light chain (MLC) (1, 2). MLC kinase and MLC phosphatase have been considered as playing a critical role in regulating endothelial cell contraction through phosphorylation/dephosphorylation of myosin (6, 7). Some recent studies indicate that RhoA and its downstream target, Rho kinase, may also increase phosphorylation of MLC by inhibiting MLC phosphatase activity (5, 8). Several proinflammatory mediators, including thrombin, histamine, cytokines and oxygen radicals, have been shown to activate MLC kinase and RhoA/Rho kinase, induce stress fiber formation, and enhance contractility of endothelial cells causing an increase in endothelial paracellular permeability (9–14). Another line of evidence indicates that, rather than contractility, it is disruption or rearrangement of the actin cytoskeleton that plays a critical role in modulating endothelial permeability (15, 16).

Morphological alterations also occur at the level of the junction complexes between adjacent endothelial cells (1, 2). Despite a growing body of evidence on how the paracellular route is opened during pathological conditions, there are still unresolved issues. Thus, it is still uncertain whether redistribution of tight and adherens junction proteins is connected with specific protein phosphorylation or dephosphorylation or whether redistribution of tight junction (TJ) proteins and actin cytoskeleton reorganization is associated with disruption of tight or adherens junctions (2, 3, 12, 17). Among the signaling molecules that affect junction coupling, protein kinase C (PKC; particularly PKCα and PKCβ), phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and the nonreceptor tyrosine kinase Src family (c-Src, Lyn, Fyn) serve as common signals for junction disorganization redistribution and as regulators of endothelial paracellular permeability (2, 18–21).

The chemokine monocyte chemoattractant protein-1 (MCP-1 or CCL2) is one of the most potent chemoattractants for monocytes during inflammation (22, 23). We recently found that CCL2, viaCCR2 receptors on brain endothelial cells, increases blood-brain barrier (BBB) permeability in vivo and in vitro (14, 24). Enhanced BBB permeability may cause increased monocyte migration and vasogenic brain edema, events that contribute to brain injury in a variety of pathophysiological states (25, 26). CCL2 causes reorganization of the endothelial actin cytoskeleton (stress fiber formation) and redistribution of TJ proteins (occludin, ZO-1, ZO-2, and claudin-5) (14). The effects of CCL2 on brain endothelial permeability are dependent upon activation of the RhoA/Rho kinase pathway. Interestingly, inhibition of RhoA or Rho

* This work was supported by National Institutes of Health Grant NS044907 (to A. V. A.).

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2 The abbreviations used are: MLC, myosin light chain; mBMEC, murine brain microvascular endothelial cell(s); DMEM, Dulbecco’s modified Eagle’s medium; PKC, protein kinase C; BBB, blood-brain barrier; TJ, tight junction; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone; PECAM, platelet/endothelial cell adhesion molecule; siRNA, small interfering RNA; TEER, transendothelial electrical resistance; DN, dominant negative.
kinase activity prevents both the reorganization of the actin cytoskeleton and the redistribution of TJ proteins (14). Because of those results, the present study examined the signaling events triggered by CCL2 in order to induce morphological and biochemical alterations in tight junction complexes associated with reorganization of the endothelial cytoskeleton.

Our results show that CCL2 activates PKC (particularly PKCα) in brain endothelial cells, which in turn induces phosphorylation of TJ proteins (occludin, ZO-1, ZO-2, claudin-5). However, PKCα activation in brain endothelial cells was strongly dependent on RhoA activation, indicating that RhoA is a crucial component in the cascade of signal events induced by CCL2.

EXPERIMENTAL PROCEDURES

Materials—CD-1 mice were obtained from Charles River (Portage, MI). Monocyte chemoattractant protein-1 (CCL2) was from Peprotech (Rocky Hill, NJ). All chemicals other than those listed below were from Sigma.

For Western blot analysis and immunocytochemistry the following antibodies were used. For TJ proteins, mouse anti-occludin, anti-ZO-1, and anti-claudin-5 antibodies were from Zymed Laboratories Inc. (Carlsbad, CA), and mouse monoclonal anti-ZO-2 antibody was from BD Biosciences. For signaling pathways, rabbit anti-Rho antibody (Upstate, Charlotteville, VA), a phosphoserine/threonine antibody sampler kit (Cell Signaling Technology Inc., Beverly, MA), anti-phospho-PKCα, -PKCβ, -PKCγ, -PKCδ, -PKCθ, -PKCα/ζ, and -PKCμ antibodies (Cell Signaling Technology Inc, Beverly, MA), and corresponding anti-PKC antibodies (BD Biosciences) were used. An anti-PECAM-1 antibody (BD Biosciences) was used. For TJ proteins, mouse anti-occludin, anti-ZO-1, anti-claudin-5, and anti-ZO-2 antibodies were from Zymed Laboratories Inc. and anti-rabbit antibodies were used. For TJ proteins, mouse anti-occludin, anti-ZO-1, anti-claudin-5, and anti-ZO-2 antibodies were from Cell Signaling Technology Inc, Beverly, MA, and anti-mouse and anti-rabbit antibodies conjugated either with fluorescein isothiocyanate or Texas Red (Vector Laboratories, Burlingame, CA).

Aprotinin, leupeptin, pepstatin A, and antipain were purchased from Roche Applied Science. For permeability experiments, [14C]inulin was obtained from PerkinElmer Life Sciences. Rho activation and PKC assay kits along with C3 exoenzyme were obtained from Upstate. The Rho kinase inhibitor Y27632 was purchased form Calbiochem. A Pierce assay kit was used for protein determination (Pierce Biotechnology). Western blots were visualized with a chemiluminescent HRP substrate kit (Pierce Biotechnology).

For cell culture preparation, the following reagents were used: Hanks’ balanced salt solution (HBSS), Dulbecco’s modified Eagle’s medium (DMEM), 10% inactivated fetal calf serum, HEPES, glutamine, antibacterial/antimycotic (Invitrogen), and dextran (60–90,000 kDa; USB Corp., Cleveland, OH). Percoll, (Amersham Biosciences), collagenase/dispase (Roche Applied Science), DNase I, TLCK, heparin (Sigma), and endothelial growth factor supplement (BD Biosciences) were also used.

Brain Endothelial Cell Cultures—Mouse brain microvascular endothelial cells (mBMEC) were prepared using a previously described protocol (14, 24). Briefly, 4–6-week-old CD-1 mice were euthanized by decapitation under isoflurane anesthesia. The brains were minced in HBSS and gently homogenized in a Dounce-type homogenizer. The homogenates were suspended in 18% dextran solution and centrifuged to remove myelin. The resulting pellet was resuspended in HBSS, layered over a preformed Percoll gradient, and centrifuged at 2700 rpm for 11 min. After centrifugation, the top layer containing the microvessels was collected and then digested for 40 min at 37 °C in HBSS solution containing 1 mg/ml collagenase/dispase, 10 units/ml DNase I, and 1 μg/ml TLCK. mBMEC were cultured in DMEM supplemented with 10% inactivated fetal calf serum, 2.5 μg/ml heparin, 20 mM HEPES, 2 mM glutamine, 1× antibiotic/antimycotic, and endothelial cell growth supplement and grown in 6-well plates coated with collagen type IV (BD Biosciences). Application of this protocol typically produces primary endothelial cell cultures that are ~99% pure (as determined by immunochemistry with an anti-PECAM-1 antibody).

Cell Transfection—To block the activity of Rho kinase, pKD-ROKα/ROKII-v6 (RokaROKII-ii sRNA expression plasmid) (Upstate) was introduced to cells. To specifically inhibit the activity of RhoA, the dominant negative mutant pCMV RhoT19N (Upstate) was introduced to mBMEC. For inhibition of PKCα and PKCζ activity, mBMEC were transfected with the dominant negative mutants pHACE-PKC-αDN and pHACE-PKC-ζDN, respectively (kindly provided by Dr. Sho, Columbia University, New York). Briefly, confluent cultures of mBMEC were transiently transfected with plasmid pCMV RhoT19N (1 μg/ml), pHACE-PKC-αDN, pHACE-PKC-ζDN (0.5 μg/ml), or empty pCMV or pHACE vector in Opti-MEM serum-deprived medium supplemented with Lipofectin, 10 μg/ml (Invitrogen) for 6 h. For the pKD-ROKα/ROKII-v6 (5 μg/ml) and pKD-NegCon-v1 (5 μg/ml) used as control, transfection was done in DMEM serum-free medium supplemented with FuGENE 6 transfection reagent (Roche Applied Science) for 48 h. The medium was then replaced with growth mBMEC medium. Twenty-four hours later experiments were performed. Transfection efficiency was evaluated by Western blot analysis.

Western Blotting—Protein concentrations were determined using a Pierce protein assay kit. Equal amounts of protein samples were loaded, separated using 7.5 and 15% SDS-polyacrylamide gel electrophoresis, and then transferred to Trans-Blot nitrocellulose membrane. Immunoblotting was performed with mouse anti-occludin, anti-ZO-1, anti-claudin-5, and anti-ZO-2 antibodies and rabbit anti-Rho, anti-PKCα, PKCβ, PKCγ, PKCδ, PKCθ, PKCα/ζ, and PKCμ along with the corresponding anti-phospho-PKC antibodies. Immunoblots were then exposed to secondary anti-mouse or anti-rabbit HRP-conjugated antibody and visualized using a chemiluminescent HRP substrate kit. The relative densities/volumes of the bands on the film negatives were measured using Image J software (NIH Image, Bethesda, MD).

Fractional Analysis of Tight Junctions—mBMEC were subjected to modified extraction protocol using a ProteoExtract subcellular proteome extraction kit (Calbiochem) according to the manufacturer’s suggestions. Utilizing different extraction buffers in the kit, four different fractions were separated: Triton-soluble cytosol fractions, Triton-insoluble membrane/organelle fractions, nuclear fractions, and actin cytoskeleton fractions. To prepare “total cell lysate” samples, cells were washed in phosphate-buffered saline, scraped, rinsed in 1 ml of radioimmunoprecipitation buffer, sonicated, and centrifuged briefly. After that, the supernatant was collected and stored at −70 °C.

Immunoprecipitation—Immunoprecipitation was performed using a protein A size exclusion immunoprecipitation kit (Sigma) according to the manufacturer’s suggestions.

Immunofluorescence—For TJ proteins, mBMEC were fixed in 4% paraformaldehyde for 20 min at 20 °C and then preincubated with a blocking solution of 5% normal goat serum, 0.05% Tween in phosphate-buffered saline. Cells were then incubated overnight in primary antibody (mouse anti-occludin, anti-ZO-1, anti-claudin-5, and anti-ZO-2) at 4 °C. Reactions were visualized by fluorescein-conjugated anti-mouse or anti-rabbit antibodies. All samples were viewed on a confocal laser-scanning microscope (LSM 510 Zeiss, objective 40×/1.3 NA).

Transendothelial Electrical Resistance (TER)—Electrical resistance across endothelial cells monolayers was measured by Millipore (World Precision Instruments). In these sets of experiments, mBMECs were plated in Transwell culture dishes (0.4-μm pore size; Corning Inc.). CCL2 (100 ng/ml) was placed in the lower and upper compart-
ment of the Transwell dual chamber system. TEER was measured between 15 min and 2 h. The resistance of blank filters was subtracted for calculation of final TEER values (ohm cm\(^2\)).

**mBMEC Monolayer Permeability**—The effects of CCL2 on endothelial monolayer permeability was examined using \([^{14}C]\)inulin, a tracer that crosses the endothelium by passive diffusion (27). Cells were cultured on Transwell culture dishes with 0.4-m pore size filters. The permeability experiments were initiated by the addition of 0.2 Ci of the isotope to the apical or donor chamber, which contained 0.4 ml of DMEM. The basal or receiving chamber contained 1.2 ml of DMEM. 0.2 ml of medium from the basal chamber was sampled and replaced with fresh DMEM at 15-min intervals from 0 to 120 min. Scintillation fluid was added to the samples, and radioactivity was counted using a Beckman 3801 liquid scintillation counter (Fullerton, CA). The permeability (\(P\)) (cm/min) of the monolayer during any time interval (\(T\)) was calculated using the following equation,

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P = \frac{(C(B)_T - C(B)) \times V(B) \times 2}{(C(A) + C(A)_T) \times A \times T} \quad \text{(Eq. 1)}\]

where \(C(B)\) and \(C(B)_T\), are, respectively, the concentrations of isotope in the basal chamber at the start and at the end of the time interval (in dpm/ml) and \(V(B)\) is the volume of the basal chamber (in ml). \(C(A)\) and \(C(A)_T\), are, respectively, the concentrations of isotope in the apical or donor chamber at the start and at the end of the time interval (in dpm/ml), and \((C(A)_T + C(A)_{T+1})/2\) is the average concentration over the time interval. \(T\) is the duration of the time interval in minutes, whereas \(A\) is the area of the filter (cm\(^2\)).

**Rho and Rho Kinase Activation Assay**—Affinity precipitation of lysed mBMEC with agarose-bound recombinant Rhotekin protein was performed according to the manufacturer’s instructions. After agarose bead removal, samples were resuspended in buffer and processed for Western blot using a rabbit polyclonal anti-Rho antibody. A Rho kinase activation assay (ROK/ROCK-II KinEASE™ FP fluorescein green assay; Upstate) was performed according to the manufacturer’s instructions.

**PKC Activation Assays**—Because some studies have shown that phospho-PKC antibodies have limitations for detecting PKC activity, we also included functional assays for PKC activity and/or specific PKC isofrom activity in total cell lysates after treatment with CCL2 (28). The following PKC assays were performed according to the manufacturer’s suggestions: colorimetric PKC assay (PepTag; Promega, Madison, WI) and specific PKC\(\alpha\) and PKC\(\xi\) assays (PKC\(\alpha\) KinEASE™ FP fluorescein green assay and PKC\(\xi\) KinEASE™ FP fluorescein green assay, Upstate).
**RESULTS**

**CCL2-induced Phosphorylation of Serine/Threonine Residues on Tight Junction Proteins**—To elucidate how CCL2 alters brain endothelial cell TJ structure, TJ protein phosphorylation was examined. mBMEC were exposed to CCL2 (100 ng/ml), and the phosphorylation status of TJ proteins in different cell fractions (denoted as Triton X-100-soluble cytosol, Triton X-100-insoluble membrane, and actin cytoskeleton fractions) was determined. As shown in Fig. 1, CCL2 induced a redistribution of TJ proteins. In cells not treated with CCL2, occludin, claudin-5, ZO-1, and ZO-2, and then these immune complexes were immunoblotted with phospho-PKCα and -PKCζ antibodies.

As shown in Fig. 1G, this analysis indicated two important things. (a) TJ proteins (occludin, ZO-1 ZO-2, and claudin-5) underwent de novo phosphorylation in the presence of CCL2, (cytosol- and cytoskeleton-insoluble fractions but not the membrane fraction). (b) Phosphorylation of these proteins occurred on Ser residues that have been described as substrates for PKC activity (29–31), suggesting that the PKC pathway could be also involved in the processing of CCL2-induced tight junction redistribution.

**CCL2 Activates Distinct PKC Isoforms in Brain Endothelial Cells**—Using a PKC activation assay, we found that CCL2 (100 ng/ml) significantly increased PKC activity in brain endothelial cells (total cell lysate), with peak activity at 10–30 min (Fig. 2A). In particular, CCL2 activated three PKC isoforms, PKCα/β, PKCθ, and PKCζ/ε (Fig. 2B). To examine whether activation of PKCα/β, PKCθ, and PKCζ/ε by CCL2 was associated with tight junction redistribution, immunoprecipitation of Triton X-100-soluble cytosol and Triton X-100-insoluble actin cytoskeleton fractions was performed with anti-occludin, ZO-1, ZO-2, and claudin-5 antibodies followed by Western blot analysis of these samples using anti-phospho-PKCα, -PKCθ, or -PKCζ antibodies. The results indicated that PKCα and PKCζ were mostly present in the cytosolic fraction (CF), whereas the actin-cytoskeleton-insoluble fraction (ACF) contained notably less amounts of PKCζ and very low levels of PKCα (Fig. 2C). On the other hand, we were not able to detect the presence of PKCθ in any of these fractions, suggesting that PKCθ is not associated with phosphorylation of TJ proteins in CCL2-induced brain endothelial hyperpermeability (data not shown). Our results, however, suggested that two PKC isoforms (PKCα and PKCζ) could participate in phosphorylation during TJ redistribution.
To further evaluate the contribution of PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 in CCL2-induced brain endothelial hyperpermeability, experiments were performed in which the activity of PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 in brain endothelial cells was diminished by transfection with dominant negative mutants for PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256. At the functional level, in cells not treated with these mutants CCL2-induced increases in \([14C]\)Inulin permeability and decreases in TEER. Both of these effects were diminished when the activity of PKC\textsuperscript{α}/H9251 or PKC\textsuperscript{ζ}/H9256 was inhibited (Fig. 3, A and B). These functional data were corroborated by biochemical analysis showing that reducing PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 activity during CCL2 treatment reduced the redistribution of occludin, ZO-1, ZO-2, and claudin-5 as evaluated by Western blot analysis (Fig. 3, C).

At the morphological level, in cells not treated with these mutants CCL2 induced a fragmentation and loss of TJ protein immunostaining (Fig. 4). In contrast, CCL2 induced only slight changes in TJ protein distribution when PKC\textsuperscript{α}/H9251 or PKC\textsuperscript{ζ}/H9256 activity was inhibited (Fig. 4). Taken together, the functional, biochemical, and morphological studies indicate that PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 are involved in the redistribution of occludin, ZO-1, ZO-2, and claudin-5 by CCL2.

**Interactions between Rho and PKCα/PKCζ in CCL2-induced Tight Junction Opening**—Prior evidence indicates that RhoA and Rho-associated kinase are involved in CCL2-induced TJ alterations as well as in the reorganization of the actin skeleton (14, 24). The data presented above indicate that two isoforms of PKC (PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256) also play a prominent role in the phosphorylation/redistribution of TJ proteins. To further reconstruct the signaling pathways triggered by CCL2, potential interactions between Rho and PKC\textsuperscript{α}/PKC\textsuperscript{ζ} in the regulation of brain endothelial permeability were studied. The involvement of PKC isoforms (PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256) in CCL2-induced increases in brain endothelial permeability and redistribution of TJ proteins was evaluated. mBMEC monolayers were first transfected either with the dominant negative mutant PKC\textsuperscript{α}/DN or PKC\textsuperscript{ζ}/DN or just with vector. Twenty-four hours later, they were exposed to CCL2 (0–120 min). Controls were not treated with CCL2. The cells were used to examine TEER at 2 h (A) and transendothelial permeability coefficient (PC) for \([14C]\)inulin from 0 to 120 min (B). Data represent mean ± S.D. of five independent experiments. * and ** indicate significant differences from CCL2-treated group at the \(p < 0.01\) and \(p < 0.001\) level, respectively. Reducing PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 activity reduced CCL2-induced barrier disruption as assessed by TEER and permeability measurements. C, reducing PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 activity in mBMEC with PKC\textsuperscript{α}/DN or PKC\textsuperscript{ζ}/DN also diminished TJ (occludin, claudin-5, ZO-1, ZO-2) redistribution from membrane (MF) or cytosol (CF) fraction to the Triton X-100-insoluble actin cytoskeleton fraction (ACF) 2 h after CCL2 treatment.

**FIGURE 3.** The involvement of PKC isoforms (PKC\textsuperscript{α} and PKC\textsuperscript{ζ}) in CCL2-induced increases in brain endothelial permeability and redistribution of TJ proteins. mBMEC monolayers were first transfected either with the dominant negative mutant PKC\textsuperscript{α}/DN or PKC\textsuperscript{ζ}/DN or just with vector. Twenty-four hours later, they were exposed to CCL2 (0–120 min). Controls were not treated with CCL2. The cells were used to examine TEER at 2 h (A) and transendothelial permeability coefficient (PC) for \([14C]\)inulin from 0 to 120 min (B). Data represent mean ± S.D. of five independent experiments. * and ** indicate significant differences from CCL2-treated group at the \(p < 0.01\) and \(p < 0.001\) level, respectively. Reducing PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 activity reduced CCL2-induced barrier disruption as assessed by TEER and permeability measurements. C, reducing PKC\textsuperscript{α}/H9251 and PKC\textsuperscript{ζ}/H9256 activity in mBMEC with PKC\textsuperscript{α}/DN or PKC\textsuperscript{ζ}/DN also diminished TJ (occludin, claudin-5, ZO-1, ZO-2) redistribution from membrane (MF) or cytosol (CF) fraction to the Triton X-100-insoluble actin cytoskeleton fraction (ACF) 2 h after CCL2 treatment.

**FIGURE 4.** Effect of PKC\textsuperscript{α} and PKC\textsuperscript{ζ} inhibition on CCL2-induced morphological alterations in brain endothelial TJ complexes. Confluent mBMEC monolayers were first transfected either with dominant negative mutant PKC\textsuperscript{α}/DN or PKC\textsuperscript{ζ}/DN or just vector. Twenty-four hours later they were exposed to CCL2 for 120 min. The cells were then fixed and processed for immunofluorescent examination of occludin, claudin-5, ZO-1, ZO-2, and ZO-3 in the presence of CCL2. Arrows indicate fragmentation and loss of immunostaining for TJ proteins. Scale bar, 20 μm.
interactions between the Rho/Rho kinase and PKC pathways were examined; i.e., do they act simultaneously and independently or is there “cross-talk” between Rho/Rho kinase and PKC/PKC\(\alpha\)/PKC\(\zeta\), where one might be a downstream target of the other? Experiments were performed in which Rho and Rho kinase activity was blocked (by dominant negative mutant RhoAT19N or with siRNA Rho kinase (pKD ROK\(\alpha\)/ROCK-II-v6)) and activity of PKC\(\alpha\) and PKC\(\zeta\) was observed by specific PKC assay or the appearance of phosphorylated PKC in brain endothelial cells. Similar experiments were performed in the opposite direction where dominant negative mutants of PKC\(\alpha\) and PKC\(\zeta\) were used to inhibit activity, and Rho and Rho kinase activity was observed. The results showed that diminishing the activity of PKC\(\alpha\) or PKC\(\zeta\) did not interrupt any changes in activity of RhoA or Rho kinase in the presence of CCL2 (Fig. 5, A–D). However, diminishing the activity of RhoA significantly diminished PKC\(\alpha\) activation, whereas PKC\(\zeta\) was unaffected. Inhibition of Rho kinase activity using the siRNA Rho kinase (pKD ROK\(\alpha\)/ROCK-II-v6) did not alter PKC\(\alpha\) and PKC\(\zeta\) activity in mBMEC treated with CCL2 (Fig. 6, A–D).

Thus, it appears that in CCL2-induced brain endothelial hyperpermeability, RhoA acts as an upstream signal molecule that activates both PKC\(\alpha\) (which causes phosphorylation and redistribution of TJ proteins) and Rho kinase (which is involved in the redistribution of TJ proteins).
and reorganization of the actin cytoskeleton. To confirm that the interaction of Rho and PKCα is critical for redistribution/phosphorylation of TJ proteins, we performed single and double transfection studies utilizing the RhoAT19N mutant and the dominant negative PKCα-DN mutant. Absence of activity of both of these two signal molecules completely blocked the effect of CCL2 on brain endothelial permeability at the functional level (particularly the permeability coefficient for fluorescein isothiocyanate-albumin). At the morphological level, transfection with the two mutants prevented the fragmentation and loss of TJ protein immunostaining that was found in control brain endothelial cells treated with CCL2 (Fig. 7, A and B). Comparing double transfection data with results obtained from inhibiting Rho or PKCα activity alone indicated that Rho acts as a critical factor for PKCα activation. Thus, blocking Rho activity alone had an effect similar to that of blocking both Rho and PKCα activity and Rho acting upstream of PKCα in regulation of permeability brain endothelial barrier by CCL2.

DISCUSSION

Previous studies have shown that the chemokine CCL2 can increase blood-brain barrier permeability in vivo and brain endothelial barrier permeability in vitro (14, 24). CCL2 alters TJ complex structure and induces actin cytoskeleton reorganization. The Rho/Rho kinase signal pathway has a prominent role in CCL2-induced hyperpermeability (14). The present study further examined the signaling pathways activated by CCL2 to enhance barrier permeability. The results demonstrate the following. (a) CCL2 causes TJ protein phosphorylation (occludin, ZO-1, ZO-2, and claudin-5) in brain endothelial cells. This occurred mostly at Ser/Thr residues that are specific substrates for PKC activity. (b) PKC isoforms (particularly PKCα and PKCζ) are activated by CCL2 and their active form is coupled to TJ proteins. (c) With respect to CCL2-induced hyperpermeability, the Rho/Rho kinase and PKC signal pathways interact at the level of Rho protein. (d) Rho proteins are key regulators of PKCα activity but do not influence PKCζ activity. The implications of these findings are discussed below.

CCL2 is the most commonly expressed chemokine during central nervous system inflammation. It is robustly expressed during the acute inflammatory response associated with ischemia/reperfusion (stroke) injury as well as in neuropathological conditions associated with so-called chronic central nervous system inflammatory responses, such as multiple sclerosis, human immunodeficiency virus infection, Alzheimer disease, and brain trauma (32–36). Mice lacking CCL2 (CCL2−/−) or the CCL2 receptor, CCR2 (CCR2−/−), have a decreased inflammatory...
response and leukocyte migration after middle cerebral artery occlusion and experimental allergic encephalitis, as well as reduced BBB disruption and brain edema formation (37, 38).

In general, BBB disruption/disregulation during central nervous system inflammation is believed to result from the “loosening” of junctional complexes between brain endothelial cells. This leads to the formation of a paracellular route that facilitates the entry of leukocytes into the brain parenchyma. Although some other pathways for leukocyte transmigration have been proposed (e.g. transcytosis), evidence from experimental and clinical studies still point to the importance of the paracellular route in leukocyte entry and BBB disruption during central nervous system inflammation (39, 40).

A number of experimental interventions and pathophysiological states can alter paracellular permeability; modify the expression, cellular distribution, and/or phosphorylation of TJ-associated proteins; and further change the functional interactions between TJ proteins and the cytoskeleton (1, 2). TJ proteins (e.g. occludin, ZO-1, ZO-2, and claudin-5) are phosphoryproteins, and regulation of their function is mostly via alterations in their state of phosphorylation (12, 41–44). There is considerable controversy over whether additional phosphorylation or dephosphorylation of TJ proteins is linked to their redistribution during increases in endothelial barrier permeability (40, 42). Changes in TJ phosphorylation and dephosphorylation status depend upon the type of cell (endothelial or epithelial), the type of stimulus (e.g. inflammatory cytokines, oxidative stress or growth factors), and the amino acid residues where phosphorylation is taking place (Ser, Thr, or Tyr). For example, vascular endothelial growth factor induces Ser/Thr phosphorylation and redistribution of occludin and ZO-1 in bovine aortic and retinal endothelial cells, but during calcium depletion, phorbol ester treatment, and bacterial infection, occludin undergoes dephosphorylation during TJ disruption (42, 45–48). The current study suggests that CCL2 acts in a manner similar to vascular endothelial growth factor, inducing phosphorylation of Ser/Thr residues on occludin, ZO-1, ZO-2, and claudin-5. This phosphorylation may affect the detergent solubility of these proteins, resulting in a shift from the Triton X-100-soluble to the Triton X-100-insoluble fraction. Phosphorylation of occludin, ZO-1, ZO-2, and claudin-5 was found in the cytosol-soluble fraction soon after CCL2 exposure followed by a further shift to the Triton-X100-insoluble actin cytoskeleton fraction. Taking into consideration that CCL2 induced a loss and fragmentation of immunostaining for TJ proteins, the shift to the actin cytoskeleton-insoluble fraction could be an indication of a possible association of these proteins with some vesicular structure (calveole or pinocytic vesicle). In epithelial cells, endocytosis appears to be involved in the redistribution of TJ proteins and barrier opening (49, 50). The role of endocytosis in the redistribution of TJ proteins at the BBB requires further investigation.

Tight junctions are regulated by a diverse group of extracellular stimuli that initiate many intracellular signaling cascades (2, 12, 51, 52). The mechanistic links between the signaling pathways and enhanced permeability have yet to be elucidated. Our previous study indicated that the CCL2-induced brain endothelial barrier “opening” is closely associated with activation of the Rho/Rho kinase axis (14). However, the current analysis of the phosphorylation status of occludin, ZO-1, ZO-2, and claudin-5 showed that CCL2 phosphorylated these proteins on Ser residues. Such residues are substrates for PKC action, suggesting that PKC could also be involved in TJ protein redistribution/phosphorylation. Our study found that two specific PKC isoforms, PKCα and PKCζ, are activated by CCL2. Both isoforms impacted upon the brain endothelial barrier permeability. PKCα has a prominent role in endothelial TJ complex assembly/disassembly. Two classic forms of PKC, PKCα and PKCβ (activated by H2O2, thrombin, and glucose), as well as atypical forms of PKC (PKCζ and PKCα), which constitute part of tight junction complex, are thought to be mostly involved in TJ disassembly (18, 31, 54, 56). In our model system, CCL2 induced activation of PKCα and PKCζ and through them caused morphological, biochemical, and functional alterations in the brain endothelial barrier. The inhibition study (specific exclusion of PKCα and PKCζ activation by transfection of brain endothelial cells with dominant negative mutants) clearly indicates the obligatory role of these two PKC isoforms in the regulation of permeability by CCL2. Under our experimental conditions, CCL2 exerts some of the effects and signaling patterns described for vascular endothelial growth factor, interleukin-6, thrombin, and bradykinin (54, 57–59). However, it
is important to note that different PKC isoforms may be activated in different model systems, making it difficult to extrapolate the role of PKC isoforms from one system to another.

The basic question addressed by our study is how does CCL2 alter TJ structure and BBB permeability? Taking into consideration the previously published data (14) and the current results, it appears that two signal pathways are involved, Rho/Rho kinase and PKC. Both pathways have the same end points, phosphorylation of TJ proteins and redistribution of those proteins away from the brain endothelial cell border. There is prior evidence that these two pathways can interact to regulate cell function. For example, in Jurkat cells, Rho interacts with PKCa and as a result activates transcription factor AP-1 (60). Similarly, an interaction of the Rho GTPase Cdc42 with atypical PKCa and PKCζ can cause stress fiber formation (56).

In conclusion, this study provides a possible signal mechanism for altering the junctional complexes between endothelial cells, which may regulate intercellular adhesion, we also would like to point out that part of the suggested signal mechanism also could be involved in the parallel process of actin cytoskeleton reorganization and increased intraendothelial contractility. However, in our opinion, Rho appears to be a critical molecule in the regulation of brain endothelial permeability, regulating both of these ongoing processes and directly modulating paracellular cleft formation between brain endothelial cells. Further studies of the specific signaling events associated with enhanced brain endothelial permeability may be of great potential therapeutic benefit, especially in conditions in which the blood-brain barrier is disrupted.

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FIGURE 8. Possible scenario of the signaling events induced by CCL2 in brain endothelial cells that result in altered paracellular permeability.
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