Protein Kinase C-\(\alpha\) Signals Rho-Guanine Nucleotide Dissociation Inhibitor Phosphorylation and Rho Activation and Regulates the Endothelial Cell Barrier Function*

Dolly Mehta‡, Arshad Rahman, and Asrar B. Malik

From the Department of Pharmacology, The University of Illinois College of Medicine, Chicago, Illinois 60612

The Rho-GDP guanine nucleotide dissociation inhibitor (GDI) complexes with the GDP-bound form of Rho and inhibits its activation. We investigated the role of protein kinase C (PKC) isozymes in the mechanism of Rho activation and in signaling the loss of endothelial barrier function. Thrombin and phorbol 12-myristate 13-acetate induced rapid phosphorylation of GDI and the activation of Rho-A in human umbilical venular endothelial cells. Inhibition of PKC by chelerythrine chloride abrogated the thrombin-induced GDI phosphorylation and Rho activation. Depletion of PKC prevented the thrombin-induced GDI phosphorylation and Rho activation, thereby indicating that these events occurred downstream of phorbol ester-sensitive PKC isozyme activation. The depletion of PKC or inhibition of Rho by C3 toxin also prevented the thrombin-induced decrease in transendothelial electrical resistance (a measure of increased transendothelial permeability), thus indicating that PKC-induced barrier dysfunction was mediated through Rho-dependent pathway. Using inhibitors and dominant-negative mutants, we found that Rho activation was regulated by PKC-\(\alpha\). Moreover, the stimulation of human umbilical venular endothelial cells with thrombin induced rapid association of PKC-\(\alpha\) with Rho. Activated PKC-\(\alpha\) but not PKC-\(\epsilon\) induced Rho phosphorylation of GDI in vitro. Taken together, these results indicate that PKC-\(\alpha\)-dependent signaling is critical in regulating GDI phosphorylation, Rho activation, and in signaling Rho-dependent endothelial barrier dysfunction.

The vascular endothelium consisting of the monolayer of endothelial cells and the extracellular matrix represents the major barrier for the exchange of liquid and solutes across the vessel wall (1–4). Thrombin by binding to the endothelial cell surface protease-activated receptor-1 induces a repertoire of signaling events that result in the development of minute gaps among cells, thereby mediating increased vascular permeability, a hallmark of tissue inflammation (5–7). Loss of endothelial barrier function primarily occurs as a result of cell shape change via actinomyosin driven contraction activated by myosin light chain phosphorylation and actin polymerization (3, 8–11).

Studies have shown an important role of the small GTPase, Rho, in the regulation of cytoskeletal dynamics, actin stress fiber formation, and myosin light chain-phosphorylation, and thus by inference, in the control of endothelial barrier function (11–13). The multiple functions of Rho are mediated through the tightly regulated GTP-binding/GT-Pase cycle (14–16). Three different classes of proteins are required for this regulation: (i) guanine nucleotide exchange factors (GEFs),\(^1\) which stimulate the GTP-GDP exchange reaction; (ii) GTPase-activating proteins (GAPs), which stimulate the GTP-hydrolytic reaction; and (iii) guanine nucleotide dissociation inhibitors (GDIs), which by binding to Rho block the dissociation of GDP from Rho GTPases (17). Furthermore, GDI is also capable of inhibiting GTP hydrolysis by Rho family GTPases as well as stimulating the release of Rho-GTPases from cellular membranes, thereby shutting off the Rho cycle (18). Thus, GDI plays a critical role in the signaling events regulated by Rho-GTPases (19).

The GDP-bound form of Rho complexed with GDI is not activated by Rho-GEFs, suggesting that Rho activation critically depends upon upstream factors mediating the dissociation of GDI from Rho (19–21). The mechanisms activating the dissociation of Rho-GDI from the Rho-GDP complex remain to be determined. It has been suggested that the dissociation of Rho-GDI might be facilitated by members of ezrin/radixin/moesin family of proteins (22, 23). However, Rho-GDI was found to interact only with the N-terminal fragment of radixin but not the full-length radixin, indicating the need of upstream effectors that are required to induce the unfolding of radixin (23). Furthermore, several studies indicate that the translocation and activation of ezrin/radixin/moesin proteins to the membrane are critically dependent on Rho, thereby indicating the intervention of other molecules that activate dissociation of GDI from Rho (24, 25).

Rho-GDI is a family consisting of Rho-GDI-1, Ly/D4-GDI, and Rho GDI-III. Of these, Rho-GDI is ubiquitously expressed (19, 22). The structure of Rho-GDI-1 indicates that it contains sequences for phosphorylation by serine-threonine kinases, raising the possibility that Rho-GDI is regulated by signaling mechanisms that induce its phosphorylation.

Protein kinase C (PKC) isozymes are serine-threonine kinases that induce phosphorylation of multiple proteins, which in turn regulate intracellular signaling (26). A PKC-dependent

---

\(^1\) The abbreviations used are: GEF, guanine nucleotide exchange factor; GAP, GTPase-activating protein; GDI, guanine nucleotide dissociation inhibitor; PKC, protein kinase C; HUVE cells, human umbilical venular endothelial cells; EBM, endothelial growth medium; FBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; GST, glutathione S-transferase; RBD, rhodotkin-Rho binding domain; PMSF, phenylmethylsulfonyl fluoride; SRE, serum response element; ANOVA, analysis of variance.

---

* This work was supported by the Midwest affiliate of the American Heart Association and NHLBI, National Institutes of Health Grants HL67016, HL46350, and HL45638. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Dept. of Pharmacology, The University of Illinois College of Medicine, 835 S. Wolcott Ave., Chicago, IL 60612. Tel.: 312-355-0236; Fax: 312-996-1225; E-mail: dmehta@uic.edu.

This is an Open Access article under the CC BY license.
pathway is important in the mechanism of thrombin-induced increase in endothelial permeability (3, 27–30). Because of the possibility that PKC may activate Rho by mediating Rho-GDI phosphorylation, we investigated the role of PKC in the mechanism of thrombin-induced Rho activation and in signaling the loss of endothelial barrier function in human umbilical venular endothelial (HUVE) cells. The present findings suggest the existence of a novel pathway by which thrombin can stimulate Rho activation. This pathway involves PKC-α-mediated phosphorylation of GDI, which may stimulate GDI dissociation, thereby resulting in Rho activation and increased endothelial permeability.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human α-thrombin was obtained from Enzyme Research Laboratories (South Bend, IN). HUVE cells and endothelial growth medium (EBM-2) were obtained from Clonetics (San Diego, CA). Phosphate-buffered saline (PBS) and trypsin were obtained from Life Technologies, Inc. Anti-Rho-A, anti-Rho-GDI, and anti-PKC-α, -ε, -δ, and -z polyclonal antibodies were obtained from Santa Cruz Biotechnology (San Diego, CA). Purified GST-Rho-GDI was purchased from Cytoskeleton, Inc. (Denver, CO).

**Endothelial Cell Culture**—HUVE cells were cultured in a T-75 flask coated with 0.1% gelatin in EBM-2 medium supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO2 and 95% air until they formed a confluent monolayer. Cells from each of the primary flasks were detached with 0.05% trypsin, 0.02% EDTA and resuspended in fresh culture medium (25 mM Tris, pH 7.4, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 500 mM NaCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton-X, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μM/ml of leupeptin, pepstatin A, and aprotinin). After clearing the lysate by centrifuging at 4 °C at 14,000 × g for 10 min, the lysate was incubated with anti-rabbit polyclonal Rho-GDI antibody for 1 h followed by the addition of protein A-agarose beads overnight at 4 °C. The beads were collected by centrifugation, washed 4 times with radioimmune precipitation buffer, electrophoresed on 4–15% gradient SDS-polyacrylamide gels, and transferred to nitrocellulose for visualization of GDI phosphorylation by autoradiography and for Western blotting with Rho-GDI antibody to verify equal protein loading. Specificity of the Rho-GDI antibody was confirmed by using peptide immunogen as a negative control.

**Reporter Gene Constructs, Endothelial Cell Transfections, and Luciferase Assay**—Rho has been shown to be primarily involved in agonist-induced SRE reporter gene activity (33). Therefore, we determined using the SRE reporter gene activity, the role of PKC isozymes in the mechanism of Rho activation. The pSRE-luciferase plasmid was kindly provided by Dr. T. Kozasa (University of Illinois, Chicago, IL). C3 transferase, produced by Electridium botulinum that specifically ADP-ribosylates and inhibits Rho protein (34), was purified from an Escherichia coli pGEX-2b-rcr recombinant vector expression system as described previously (35).

The expression vector pcDNA3-containing tagged dominant-negative form of PKC-α and PKC-ε isozymes were provided by Dr. I. B. Weinstein (Columbia University, New York, NY). The dominant-negative PKC-α, PKC-ε, and PKC-δ mutants lacking a functional catalytic domain were generated by a substitution of lysine 368, 437, or 376 for arginine, respectively (36). Transfections were performed with DEAE-dextran method (37). 5 μg of DNA were mixed with 50 μg/ml DEAE-dextran in serum-free EB medium, and the mixture was added onto 70–80% confluent cells. pTKRLUC plasmid (0.125 μg) (Promega Corp., Madison, WI) containing Renilla luciferase gene driven by the constitutively active thymidine kinase promoter was added to normalize the transfection efficiencies. At 70–80% confluence, cells were incubated for 4 h with 10% dimethyl sulfoxide (Me2SO) in serum-free medium, washed twice with EB containing 10% fetal bovine serum, and grown to confluence. Cells extracts were prepared and assayed for luciferase activity using the Dual Luciferase Reporter Assay System (Promega, Madison, WI). SRE-luciferase activity was expressed as the ratio of firefly and Renilla luciferase activity. Cell viability (>95%) after transfection was confirmed using trypan blue (Sigma) exclusion assay.

**Immunocomplex Protein Kinase Assay**—Phosphorylation of Rho-GDI in vitro was performed using immunocomplexes of PKC-α or PKC-ε obtained after immunoprecipitation of cell lysate with respective PKC antibodies as described previously (38). Confluent cells grown in 100-mm dishes were stimulated for the indicated times with 50 nM thrombin, washed quickly with ice-cold PBS, and lysed in radioimmune precipitation buffer containing 50 mM Tris, pH 7.4, 150 mM NaCl, 0.25 mM EDTA, pH 8.0, 1% deoxycholic acid, 1% Triton-X, 5 mM NaF, 1 mM sodium orthovanadate, 1 mM PMSF, and 5 μg/ml each of leupeptin and aprotinin, and 1 μg/ml pepstatin A. The lysate was scraped and cleared by centrifugation at 4 °C at 14,000 × g for 10 min. Cell lysate containing an equal amount of protein was then incubated with anti-rabbit polyclonal PKC-α or PKC-ε antibody for 1 h followed by an addition of protein A-agarose beads overnight at 4 °C. Beads from each sample were collected by centrifugation, washed twice with PBS containing 0.1% Triton-X 100, 0.5% sodium deoxycholate, and 0.1% SDS, 500 mM NaCl, 10 mM MgCl2, 10 μg/ml each of aprotinin and leupeptin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). Cell lysates were clarified by centrifugation at 14,000 × g at 4 °C for 2 min, and equal volumes of cell lysates were incubated with GST-RBD beads (15 μg) at 4 °C for 1 h. The beads were washed 3 times with wash buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 10 mM MgCl2, 10 μg/ml each of aprotinin and leupeptin, and 0.1 mM PMSF), and bound Rho was eluted by boiling each sample in Laemmli sample buffer. Eluted samples from beads and total cell lysate were then electrophoresed on 12.5% SDS-polyacrylamide gel electrophoresis gels, transferred to nitrocellulose, blocked with 5% nonfat milk, and analyzed by Western blotting using a polyclonal anti-Rho-A antibody. The amount of RBD-bound Rho was normalized to the total amount of Rho in cell lysates for quantitation of Rho activity in different samples using scanning densitometry.

**Phosphorylation of Rho-GDI**—A serum-starved confluent monolayer of HUVEC cells was labeled with 300 μCi/ml of [32P]ATP for 4 h in phosphate-free medium, after which they were stimulated with 50 nM thrombin or 100 nM PMA at indicated times. Cells were quickly rinsed twice with ice-cold PBS and then lysed for 20 min on ice with 300 μl of radioimmune precipitation buffer (10 mM Tris, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1% Triton-X, 1 mM sodium orthovanadate, 1 mM PMSF, and 1 μM/ml of leupeptin, pepstatin A, and aprotinin). After clearing the lysate by centrifuging at 4 °C at 14,000 × g for 10 min, the lysate was incubated with anti-rabbit polyclonal Rho-GDI antibody for 1 h followed by the addition of protein A-agarose beads overnight at 4 °C. The beads were collected by centrifugation, washed 4 times with radioimmune precipitation buffer, electrophoresed on 4–15% gradient SDS-polyacrylamide gels, and transferred to nitrocellulose for visualization of GDI phosphorylation by autoradiography and for Western blotting with Rho-GDI antibody to verify equal protein loading. Specificity of the Rho-GDI antibody was confirmed by using peptide immunogen as a negative control.
RESULTS

Thrombin and PMA Activate Rho in HUVE Cells—We used GST-rhotekin fusion protein, which specifically binds to activated Rho, to quantitate Rho activation (32, 39). Thrombin induced a 3–4-fold increase in Rho activity in a time-dependent manner with a maximum response occurring at 1 min followed by a decline at 10 min (Fig. 1, A and B). We also used PMA, a direct activator of PKC, to determine the role of PKC activation in mediating Rho activation. As shown in Fig. 1, C and D, PMA induced a 2–3-fold increase in Rho activity in a time-dependent manner with maximum activation at 10 min.

Thrombin-induced Rho Activation Is Secondary to PKC Activation—To investigate whether PKC acts upstream of Rho activation, we used chelerythrine chloride, a specific (but not isoyme-selective) PKC inhibitor belonging to a new class of PKC inhibitors that interfere with the phosphate acceptor site and non-competitively inhibit the ATP binding site (40, 41). Chelerythrine pretreatment of HUVE cells prevented thrombin-induced Rho activation, indicating that PKC is an upstream regulator of Rho activation (Fig. 2).

To determine whether PKC isozymes involved in thrombin-induced Rho activation were phosphor-sensitive, we studied the effects of PKC depletion by PMA on thrombin-induced Rho activation. HUVE cells were pretreated without or with 500 nM PMA overnight, after which they were stimulated with thrombin to measure Rho activation. Fig. 3A shows that overnight pretreatment of HUVE cells with PMA prevented Rho activation in response to thrombin. Western blot analysis of the cell lysate from these samples showed that exposure of HUVE cells to phorbol esters resulted in the depletion of PKC-α, PKC-δ, and PKC-ε with PKC-α and PKC-ε being most sensitive to phorbol esters, whereas residual levels of PKC-δ remained detectable. In contrast phorbol ester treatment had no effect on PKC-ζ (Fig. 3B). Thus, these results indicate that thrombin-induced Rho activation in HUVE cells is regulated by phorbol-sensitive PKC isozymes but not by the atypical PKC isozymes.

Using LY379196 and rottlerin, which inhibit PKC-β and PKC-δ isozymes, respectively (42–44), we ruled out the involvement of PKC-β or PKC-δ isoforms in regulating thrombin-induced Rho activation (data not shown). As these data and the results in Fig. 2 pointed to the phorbol ester-sensitive PKC isoforms, such as PKC-α, as being responsible for thrombin-induced Rho activation, we studied the specific role of PKC-α in the mechanism of thrombin-induced Rho activation.

PKC-α Mediates Rho-dependent Thrombin-induced SRE Reporter Activity in HUVE Cells—Using the SRE reporter gene activity, we determined the role of PKC isozymes in the mechanism of Rho activation. We used the dominant-negative mutant of PKC-α to address its role in thrombin-induced Rho activation. The dominant-negative mutants of PKC-δ and PKC-ε were also included in these experiments. In addition, we studied the effect of C3 transferase, which inhibits Rho activation (34), on thrombin-induced SRE activation.

HUVE cells were cotransfected with SRE-luciferase reporter gene construct together without or with C3 transferase and assayed for thrombin-induced SRE luciferase activity. Thrombin increased SRE reporter gene activity by 4-fold, whereas it
failed to increase SRE activation in HUVE cells that were cotransfected with C3 transferase. These results indicate that thrombin-induced SRE reporter gene activation is mediated by Rho (Fig. 4A).

The results of thrombin-induced SRE reporter gene activity in cells cotransfected with SRE-luciferase reporter without or with dominant-negative mutants of PKC-α, PKC-δ, and PKC-ε are shown in Fig. 4B. Thrombin-induced SRE reporter gene activity was completely prevented in cells transfected with the dominant-negative mutant of PKC-α. In contrast, dominant-negative PKC-δ or PKC-ε had no significant effect on thrombin-induced SRE activation. Thus, these results, which show that PKC-α is critical in stimulating Rho activation, are in accord with the above findings, which were obtained using pharmacological inhibitors.

**Thrombin Induces Rapid Association of Rho with PKC-α**—To determine whether thrombin-induced regulation of Rho by PKC-α occurs as a result of their physical interaction, lysates from cells stimulated without or with thrombin (1-min challenge period) were incubated with GST-rhotekin fusion protein to pull down activated Rho, after which they were subjected to Western blotting with anti-Rho, PKC-α, or PKC-ε antibody. Fig. 5 shows that thrombin induced the rapid association of PKC-α and Rho, whereas under similar conditions, Rho and PKC-ε did not associate.

**Thrombin and PMA Induces Rho-GDI Phosphorylation in HUVE Cells**—The GDP-bound form of Rho complexed with GDI is not activated by Rho-GEFs (19), suggesting that Rho activation critically depends on upstream factors that activate the dissociation of GDI. Since Rho-GDI contains sequence for phosphorylation, we addressed the possibility that the activation of HUVE cells results in the phosphorylation of GDI. Fig. 6 shows the autoradiograph of GDI phosphorylation in 32P-labeled HUVE cells in response to thrombin or PMA stimulation. Thrombin as well as PMA induced the rapid phosphorylation of GDI that returned to near basal level at 5 min after challenge (Fig. 6).

**Thrombin-induced GDI Phosphorylation Is Secondary to PKC Activation**—Since thrombin-induced Rho activation was blocked by either PKC depletion of cells or by the inhibition of PKC using chelerythrine, we determined the role of PKC in mediating thrombin-induced GDI phosphorylation. We found that depletion of PKC by overnight pretreatment with PMA abrogated thrombin-induced phosphorylation of GDI. Similarly, the inhibition of PKC by chelerythrine pretreatment prevented the phosphorylation of GDI in response to thrombin stimulation of endothelial cells. Thus, these results demonstrate that phorbol-sensitive PKC isozymes are involved in regulating the phosphorylation of GDI in response to thrombin.
PKC-α Phosphorylate Rho-GDI—As Rho activation was regulated by PKC-α, we next performed an immunocomplex protein kinase assay to determine whether PKC-α can directly phosphorylate GDI in vitro. HUVE cells were depleted of phorbol-sensitive PKC isozymes by overnight treatment with 500 nM PMA. Serum-starved cells were then labeled with 32P in phosphate-free medium. In parallel, labeled control kinase reactions were performed using immunocomplex independent experiments. Data are representative of three independent experiments. α-T, α-thrombin; P-GDI, phosphorylated GDI; −, absence; +, presence.

PKC Depletion or Rho Inhibition Inhibits Thrombin-induced Decrease in Transendothelial Electrical Resistance—The biochemical events by which PKC activation regulates thrombin-induced barrier dysfunction are incompletely understood (3, 27–30). Because the results of this study directly implicate PKC as the upstream regulator of Rho, we measured changes in transendothelial electrical resistance (the basis of increased paracellular endothelial permeability) in PKC-depleted cells or in cells treated with C3 transferase to block Rho activation. As shown in Fig. 9, thrombin caused a significant decrease in resistance in untreated cells, whereas the decreases in resistance in response to thrombin were significantly reduced in cells, which were depleted of phorbol ester-sensitive PKC isoforms, as well as in cells treated with C3 transferase.

Discussion

Rho activation plays an important role in the mechanism of increased transcellular permeability induced by mediators such as thrombin (11–13, 39); however, the mechanisms of activation of Rho, thereby the loss of endothelial barrier integrity, are not elucidated (39). The dissociation of GDI from Rho-GEF (19, 22, 45, 46). As GDI may play a critical role in mediating thrombin-induced Rho activation and thus in signal transduction, we addressed in this study the basis of Rho activation and its contribution in mediating the loss of endothelial barrier function induced by thrombin.

The present results provide several lines of evidence that Rho-GDI phosphorylation and Rho activation are regulated by a PKC-dependent pathway in endothelial cells. We showed that thrombin as well as the direct activation of PKC by PMA...
PKC-α Signals Rho-GDI Phosphorylation and Activation of Rho

induced the phosphorylation of Rho-GDI and that the Rho-GDI phosphorylation occurred concurrently with the thrombin-induced activation of Rho. Furthermore, the inhibition of PKC by chelerythrine (a specific but not isoyme-selective inhibitor of PKC) abrogated not only thrombin-induced Rho activation but also Rho-GDI phosphorylation. We also showed that the phosphorylation of GDI and Rho activation is regulated by phorbol ester-sensitive isozymes as the depletion of these isozymes by exposing HUVE cells to phorbol esters in the standard manner prevented thrombin-induced GDI phosphorylation and Rho activation. Because the PKC isozymes, α, β, δ, and ε, expressed in endothelial cells are all phorbol ester-sensitive, we used both pharmacological and genetic approaches to further identify the specific PKC isozyme regulating GDI phosphorylation and Rho activation.

We found that the treatment of HUVE cells with LY379196 or rottlerin, which inhibits PKC-β or PKC-δ isozymes, respectively, failed to prevent Rho activation in response to thrombin in endothelial cells. Using dominant-negative mutant constructs, we showed that dominant-negative PKC-δ failed to prevent thrombin-induced SRE reporter gene activity that is regulated by Rho. Furthermore, we found that Rho-mediated SRE reporter gene activity in response to thrombin was completely prevented in endothelial cells transfected with the dominant-negative mutant of PKC-α, whereas PKC-ε had no significant effect on thrombin-induced SRE activation. Thus, these data demonstrate that PKC-α is the major kinase regulating Rho activation in endothelial cells. As the above findings indicate the critical role of PKC-α activation in the regulation of Rho activation, we used in vitro kinase assay to test the possibility that PKC-α can directly phosphorylate GDI. Results of the in vitro kinase assay using PKC-α and PKC-δ immunoprecipitates from unstimulated and stimulated cells indicated that only PKC-α from activated cells was capable of inducing phosphorylation of GST-GDI. Thus, these findings indicate that the activation of PKC-α is required for phosphorylation of Rho-GDI, although the possibility cannot be ruled out that PKC may also activate another protein kinase controlling the phosphorylation state of GDI in HUVE cells.

We also found in the pull-down assay that stimulation of endothelial cells with thrombin leads to a rapid association of PKC-α with the activated Rho, although it failed to associate with PKC-ε. The association of PKC-α with Rho after activation, although not in resting cells, indicates that the protein complex formation is probably important in targeting and regulating Rho function (14, 47). However, our results do not distinguish between the possibility that association of PKC-α with Rho can occur directly or whether it is mediated by intermediate factors.

There is little information regarding the role of different PKC isoforms in regulating the activation of Rho. Studies in endothelial and epithelial cells have implicated Rho in PMA-induced recruitment of PKC-α to the cell membrane (48); however, these observations were not confirmed in bovine arterial endothelial cells (49). A permissive role of PKC-α but not PKC-δ in sphingosine 1-phosphate-induced Rho-A translocation from cytosol to membrane (an indirect measure of Rho activation) was also recently reported in C2C12 myoblasts (50). Thus, on the basis of using multiple approaches our results provide unequivocal evidence that PKC-α is a key upstream regulator of Rho activation.

Several studies have implicated a critical role of PKC-dependent pathway in regulating thrombin-induced increase in endothelial permeability (3, 27–30). We, therefore, measured changes in transendothelial electrical resistance (the basis of increased paracellular endothelial permeability) (31) using cells depleted of PKC by PMA treatment or cells treated with C3 transferase to inhibit Rho. We used these cells to address the possibility that PKC-induced barrier dysfunction can be explained by Rho activation. The results showed that thrombin caused a decrease in transendothelial electrical resistance, whereas depletion of PKC or inhibition of Rho reduced the response. Thus, these observations indicate that PKC induces the permeability increase activated by thrombin via the Rho-mediated pathway.

What are the implications of PKC-α-induced GDI phosphorylation in the mechanism of Rho activation? It has been shown that the cytoplasmic pool of Rho-GTPase is complexed with GDP- and GTP-bound states. The dissociation of GDI from the Rho protein is a prerequisite for membrane association and its activation by Rho-GEFs (19, 20). In bovine neutrophils, phosphorylation/dephosphorylation events have been implicated in the regulation of the dissociation of the Rho/Rho-GDI complex (51).

Based on our results of thrombin-induced phosphorylation of GDI and Rho activation through a PKC-dependent pathway, we hypothesize that phosphorylation/dephosphorylation of GDI may play a role in the mechanism of PKC-α-induced activation of Rho. Thus, the results of this study describe a novel pathway of GDI phosphorylation and Rho activation regulated by PKC-α and in signaling PKC-induced loss of endothelial barrier function.

Acknowledgments—We thank Dr. Martin Schwartz for providing rhotekin-Rho-binding domain construct and Dr. Tohra Kozasa for providing SRE-luciferase construct. We thank Dr. I. B. Weinstein for providing PKC-α, PKC-δ, and PKC-ε isozyme constructs. We also thank Arash Jalali for expert technical assistance and Mike Holinstat and Dr. K. Anwar for help in purifying SRE construct and luciferase activity measurements.

REFERENCES

1. Gao, B., Curtis, T. M., Blumenstock, F. A., Minnear, F. L., and Saba, T. M. (2000) J. Cell Sci. 113, 247–257
2. Lampugnani, M. G., Resnati, M., Dejana, E., and Marchisio, P. C. (1999) J. Cell Biol. 122, 479–490
3. Lum, H., and Malik, A. B. (1994) Am. J. Physiol. 267, L223–L241
4. Qiao, R. L., Yan, W., Lum, H., and Malik, A. B. (1995) Am. J. Physiol. 269, C110–C117
5. Gersten, R. E., Chen, J., Ishii, M., Ishik, W., Nanoev, T., Turkc, C. W., Wu, T. K., and Coughlin, S. R. (1994) Nature 368, 648–651
6. Garcia, J. G., Patterson, C., Bahler, C., Aicher, J., Hart, C. M., and English, D. (1993) J. Cell. Physiol. 156, 541–549
7. Lum, H., Andersen, T. S., Siffinger-Birnboim, A., Turupathi, C., Goligorsky, M. S., Fenton, J. W., and Malik, A. B. (1995) J. Cell Biol. 120, 1499–1499
8. Moy, E. B., Day, C. M., and Natarajan, V. (1993) J. Cell. Physiol. 156, 541–549
9. Garcia, J. G., Pavalko, F. M., and Patterson, C. E. (1993) Blood Coagul. Fibrinolysis 4, 609–626
10. Garcia, J. G., Davis, H. W., and Patterson, C. E. (1995) J. Cell. Physiol. 163, 510–522
11. Garcia, J. G., Verin, A. D., Schopf, K., Siddiqui, R., Patterson, C. E., Coortos, C., and Natarajan, V. (1999) Am. J. Physiol. 276, L889–L989
12. van Nieuw Amerongen, G. P., Draijer, R., Vermeer, M. A., and van Hinsbergh, V. W. (1998) Circ. Res. 83, 1113–1123
13. Esler, M., Amano, M., Kruze, H. J., Kuoibi, K., Weber, P. C., and Aepfelbacher, M. (1998) J. Biol. Chem. 273, 21867–21874
14. Hall, A. (1998) Science 280, 2074–2075
15. Sah, V. P., Sehgal, T. M., Sagi, S. A., and Brown, J. H. (2000) Annu. Rev. Pharmacol. Toxicol. 40, 459–489
16. Kabiuchi, K., Kuroda, S., and Amano, M. (1999) Annu. Rev. Biochem. 68, 459–486
17. Geyer, M., and Wittinghofer, A. (1997) Curr. Opin. Struct. Biol. 7, 786–792
18. Nomanbhoy, T. K., and Cerione, R. A. (1997) J. Biol. Chem. 272, 23371–23375
19. Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y., and Tsukita, S. (1994) J. Biol. Chem. 269, 1810–1817
20. Hart, M. J., Maru, Y., Leonard, D., Witte, O. N., Evans, T., and Cerione, R. A. (1992) Science 258, 812–815
21. Yaku, H., Sasaki, T., and Takai, Y. (1994) Biochem. Biophys. Res. Commun. 205, 812–817
22. Sasaki, T., and Takai, Y. (1998) Biochem. Biophys. Res. Commun. 245, 641–645
23. Takahashi, K., Sasaki, T., Mamamoto, A., Takaiishi, K., Kamayama, T., Tsutki, S., and Takai, Y. (1997) J. Biol. Chem. 272, 23371–23375
24. Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y., and Tsukita, S. (1996) J. Cell Biol. 135, 37–51
25. Kotani, H., Takaiishi, K., Sasaki, T., and Takai, Y. (1997) Oncogene 14,
PKC-α Signals Rho-GDI Phosphorylation and Activation of Rho

1705–1713

26. Nishizuka, Y. (1992) Science 258, 607–614
27. Vuong, P. T., Malik, A. B., Nagpala, P. G., and Lum, H. (1998) J. Cell. Physiol. 175, 379–387
28. Aschner, J. L., Lum, H., Fletcher, P. W., and Malik, A. B. (1997) J. Cell. Physiol. 173, 387–396
29. Patterson, C. E., Davis, H. W., Schaphorst, K. L., and Garcia, J. G. (1994) Microvasc. Res. 48, 212–235
30. Lynch, J. J., Ferro, T. J., Blumenstock, F. A., Brockenauer, A. M., and Malik, A. B. (1990) J. Clin. Invest. 85, 1991–1998
31. Tiruppathi, C., Malik, A. B., Del Vecchio, P. J., Keese, C. R., and Giaever, I. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7919–7923
32. Rea, X. D., Kissoses, W. B., and Schwartz, M. A. (1999) EMBO J. 18, 578–585
33. Hill, C. S., Wynne, J., and Treisman, R. (1995) Cell 81, 1159–1170
34. Paterson, H. F., Self, A. J., Garrett, M. D., Just, I., Aktories, K., and Hall, A. (1990) J. Cell Biol. 111, 1001–1007
35. Merli, N., and Nurumiya, S. (1995) Methods Enzymol. 256, 196–206
36. Sch, J. W., Lee, E. H., Prywes, R., and Weinstein, I. B. (1999) Mol. Cell. Biol. 19, 1313–1324
37. Rahman, A., Anwar, K. N., and Malik, A. B. (2000) Am. J. Physiol. 278, C906–C914
38. Jain, N., Zhang, T., Kee, W. H., Li, W., and Cao, X. (1999) J. Biol. Chem. 274, 24392–24400
39. van Nieuw Amerongen, G. P., van Delft, S., Vermeer, M. A., Collard, J. G., and van Hinsbergh, V. W. (2000) Circ. Res. 87, 335–340
40. Herbert, J. M., Augereau, J. M., Gleye, J., and Maffrand, J. P. (1990) Biochem. Biophys. Res. Commun. 172, 993–999
41. Laubhanna, C., Mohly-Rosen, D., Liron, T., Constantine, G., and Butcher, E. C. (1998) J. Biol. Chem. 273, 30306–30315
42. Jirousek, M. R., Gillig, J. R., Gonzalez, C. M., Heath, W. F., McDonald, J. H., Neel, D. A., Rito, C. J., Singh, U., Stramm, L. E., Melkian-Badalain, A., Baeysky, M., Bailas, L. M., Hall, S. E., Winneroski, L. L., and Faul, M. M. (1996) J. Med. Chem. 39, 2664–2671
43. Gschwendt, M., Muller, H. J., Kielbassa, K., Zang, R., Kittstein, W., Rincke, G., and Marks, F. (1994) Biochem. Biophys. Res. Commun. 199, 93–98
44. Pongrutz, J., Webb, P., Wang, K., Deacon, E., Lunn, O. J., and Lord, J. M. (1999) J. Biol. Chem. 274, 37329–37334
45. Regazzi, R., Kikuchi, A., Takai, Y., and Wollheim, C. B. (1992) J. Biol. Chem. 267, 17512–17519
46. Hoffman, G. R., Nassar, N., and Cerione, R. A. (2000) Cell 100, 345–356
47. Tapon, N., and Hall, A. (1997) Curr. Opin. Cell Biol. 9, 86–92
48. Hinnenstiel, S., Kratz, T., Krull, M., Seybold, J., von Eichel-Streiber, C., and Suttrop, N. (1998) Biochem. Biophys. Res. Commun. 245, 830–834
49. Verin, A. D., Liu, F., Bogatcheva, N., Borbiev, T., Hershenson, M. B., Wang, P., and Garcia, J. G. (2000) Am. J. Physiol. 279, L360–L370
50. Meseci, E., Donati, C., Cencetti, F., Romiti, E., and Bruni, P. (2000) FEBS Lett. 482, 97–101
51. Bourmeyster, N., and Vignais, P. V. (1996) Biochem. Biophys. Res. Commun. 218, 54–60