Involvement of PKCδ and PKD in pulmonary microvascular endothelial cell hyperpermeability

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Submitted 6 August 2003; accepted in final form 8 September 2003

Tinsley, John H., Nicole R. Teasdale, and Sarah Y. Yuan. Involvement of PKCδ and PKD in pulmonary microvascular endothelial cell hyperpermeability. Am J Physiol Cell Physiol 286: C105–C111, 2004. First published September 17, 2003; 10.1152/ajpcell.00340.2003.—The involvement of PKC, the isoforms of which are categorized into three subtypes: conventional (α, βI, βII, and γ), novel [δ, ε, η, and μ (also known as PKD,θ)], and atypical (ζ and ζ′), in the regulation of endothelial monolayer integrity is well documented. However, isoform activity varies among different cell types. Our goal was to reveal isoform-specific PKC activity in the microvascular endothelium in response to phorbol 12-myristate 13-acetate (PMA) and diacylglycerol (DAG). Isoform activity was demonstrated by cytosol-to-membrane translocation after PMA treatment and phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) protein after PMA and DAG treatment. Specific isoforms were inhibited by using both antisense oligonucleotides and pharmacological agents. The data showed partial cytosol-to-membrane translocation of isoforms α, βI, and γ and complete translocation of PKCδ and PKD in response to PMA. Furthermore, antisense treatment and pharmacological studies indicated that the novel isoform PKCδ and PKD are both required for PMA- and DAG-induced MARCKS phosphorylation and hyperpermeability in pulmonary microvascular endothelial cells, whereas isoforms α, βI, and ε were dispensable with regard to these same phenomena. Signal transduction; permeability; myristoylated alanine-rich C kinase substrate; microvasculature; pulmonary endothelium

An intricate monolayer of closely opposed cells, the microvascular endothelium operates as a semi-permeable barrier to regulate the transvascular passage of solutes, fluid, and blood cells. In addition to this barrier function, the endothelium participates in homeostatic mechanisms such as antithrombogenesis, angiogenesis, regulation of leukocyte dynamics, and control of tissue perfusion. Inflammatory agonists such as activated neutrophils, histamine, thrombin, and the PKC activators phorbol 12-myristate 13-acetate (PMA) and diacylglycerol (DAG) are capable of evoking intracellular signaling events that function of which is the alteration of normal endothelial operation, i.e., hyperpermeability across the cell monolayer (11, 12, 22, 23, 32).

There are currently 12 known isoforms of PKC: conventional (cPKC) isoforms (α, βI, βII, and γ), novel (nPKC) isoforms (δ, ε, η, μ, and θ), and atypical (aPKC) isoforms (ζ, ζ′, and ζ′′) (13, 15). PKCμ is often called protein kinase D (PKD), and we will hereafter refer to it as such. Activation of these three subtypes of PKC occurs under different mechanisms. The cPKC isoforms are dependent on phosphatidylserine, DAG, and Ca2+, and they are activated by PMA. The nPKC isoforms differ in that they become activated in the absence of Ca2+. The aPKC isoforms are both independent of DAG and Ca2+ and are not activated by PMA. The PKC isoforms vary widely with regard not only to cell specificity but also to subcellular localization within distinct cell types. For example, the PKC isoforms α, δ, and ε have been shown to be involved in the regulation of barrier function and tight junction permeability in epithelial cells (14, 20). PKCα, βI, and δ have been implicated in lipopolysaccharide-induced nitric oxide synthase expression in macrophages (6). Furthermore, studies in neuronal cells show that PKCζ and PKCδ are activated by different growth factors and that these processes independently result in mitogen-activated protein kinase activation (7).

Our previous studies and those of others have implicated the involvement of PKC in regulating endothelial function (1, 11, 24, 27). Much of the recent work is being devoted to elucidating the specific isoform(s) of PKC being expressed and operating in endothelial cells of macrovascular origin. For example, PKCα is known to increase endothelial permeability and is required for endothelial cell migration and vascular tube formation in human umbilical vein endothelial cells (HUVEC) (16, 29). In vivo experiments on healthy humans determined that inhibition of PKCδ attenuated endothelium-dependent vasodilation caused by experimental hyperglycemia (2). Our recent studies indicate a preferential upregulation of PKCδ gene expression in porcine cardiovascular tissues during the early development of diabetes (9). Pharmacological inhibition of PKCδ attenuates hyperglycemia-induced increases in coronary venular permeability (31). PKCζ in human pulmonary artery endothelial cells (HPAE) and PKCδ in HUVEC have been shown to regulate TNF-α-induced NADPH oxidase (8) and VEGF-induced proliferation (18). In addition, overexpression of PKCζ apparently augments the hyperpermeability response to thrombin, although PKCζ may protect against barrier dysfunction (10). This sampling of previous findings makes apparent the diverse functions controlled by various PKC isoforms in the macrovascular endothelium. However, little is known regarding the role(s) of PKCδ and, more precisely, the mechanisms regulated by distinct PKC isoforms in the microvascular endothelium, the main barrier to bloodstream/tissue exchange of fluids, solutes, and gasses.

This study focuses on the effects of PMA- and DAG-induced PKC activation and subsequent hyperpermeability response across the endothelial monolayer in rat lung micro-
vascular endothelial cells (RLMEC). Using various pharmacological inhibitors, we were able to predict which PKC isoforms were activated by PMA and responsible for permeability increases. By employing more specific means, i.e., antisense oligonucleotides directed against the different isoforms, we found that the nPKC isoform δ and PKD were necessary for alterations in endothelial barrier function as measured by albumin flux across the monolayer. Furthermore, the cPKC isoforms α and βI, as well as nPKCε, were not required for PMA- and DAG-induced endothelial hyperpermeability. In addition, of the five isoforms we were able to detect in these cells, PKCβ and PKD were the only two found to fully translocate from the cytosol to the membrane in response to PMA. Finally, elimination of PKCβ and PKD by antisense oligonucleotides attenuated the PMA- and DAG-induced phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) protein, a prominent PKC substrate (5, 25).

The PKC isoforms α, βI, and ε showed incomplete cytosol-to-membrane translocation and apparently were not required for MARCKS phosphorylation after PMA treatment. This work is the first to show that the specific PKC isoform δ and PKD are both required for pulmonary microvascular endothelial barrier disruption in response to PMA and DAG stimulation.

MATERIALS AND METHODS

Chemicals. The chemicals used were PMA, bisindolylmaleimide I (BIM), Rottlerin, G06976, and G06983 from Calbiochem (La Jolla, CA); dioctanoylglycerol (DOG, a cell-permeable diacylglycerol) was from Sigma.

Cell culture. RLMEC (VEC Technologies, Rensselaer, NY) were maintained on gelatin-coated dishes in complete MCDB-131 medium from Sigma. Cell culture. RLMEC were treated with 10% fetal bovine serum and used at maintained on gelatin-coated dishes in complete MCDB-131 medium from Sigma.

Materials and Methods. Materials and Methods. MATERIALS AND METHODS

Cell culture. RLMEC (VEC Technologies, Rensselaer, NY) were maintained on gelatin-coated dishes in complete MCDB-131 medium supplemented with 10% fetal bovine serum and used at passages 3–7. Cells were incubated at 37°C in 5% CO2. RLMEC exhibited properties characteristic of the endothelial cell, such as typical cobblestone morphology and incorporation of acetylated low-density lipoprotein.

Measurement of endothelial permeability. Measurement of endothelial permeability. RLMEC permeability was determined by measuring FITC-albumin flux across the monolayer as previously described (22, 23). Cells were grown on gelatin-coated Costar Transwell membranes (VWR, Houston, TX). All transfection reagents and chemicals were added to the luminal chamber. FITC-albumin (3 μg/ml) was added to the luminal chamber for 30 min, and samples were removed from both luminal and abluminal chambers for fluorescence analysis. The readings were converted with the use of a standard curve to albumin concentration. These concentrations were then used in the following equation to determine the permeability coefficient of albumin ($P_a$): $P_a = \frac{A}{V} \times \frac{V}{l}$, where $A$ is abluminal concentration, $V$ is volume of abluminal chamber, and $l$ is luminal concentration.

Oligonucleotide transfection. Oligonucleotide transfection. RLMEC were grown to confluence on either 60-mm dishes for Western analysis or Transwell membranes for permeability experiments. The complete medium was replaced with OPTI-MEM (Invitrogen) containing the antisense phosphorothioate oligonucleotides (5.6 μg/ml) that had been preincubated with Lipofectin (10 μg/ml) (Invitrogen) for 30 min. After 6 h of incubation at 37°C, the cells were washed and returned to complete medium containing oligonucleotide (2.9 μg/ml) for an additional 42 h before experimentation. All antisense oligonucleotides were synthesized by Sigma-Genosys (The Woodlands, TX) and HPLC purified before transfection. Sequences are shown in Table I.

Subcellular fractionation and Western analysis. Subcellular fractionation and Western analysis. Cells were treated as appropriate before examining PKC isoform translocation. After washes with cold phosphate-buffered saline (PBS), cells were suspended in 600 μl of digitonin buffer [0.55% digitonin, 25 mM Tris-HCl, pH 7.6, 5 mM EGTA, 5 mM EDTA, Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL)]. The resulting pellet was dissolved in 500 μl of digitonin buffer containing 1% Triton X-100, passed through a 26-gauge needle several times, and centrifuged again for 10 min at 10,000 g for 10 min at 4°C. The supernatant was labeled the membrane fraction. For total lysate, cells were suspended in digitonin buffer containing 1% Triton X-100, passed through a needle, and centrifuged as above with the supernatant labeled total protein.

For Western analysis, 10 μg of protein were electrophoresed by SDS-PAGE and blotted to nitrocellulose membrane. Membranes were blocked with 3% bovine serum albumin followed by exposure to primary antibodies to PKC isoforms or MARCKS protein and subsequent secondary antibody conjugated to horseradish peroxidase. Enhanced chemiluminescence (ECL) was employed to detect bands of interest. Primary antibodies (all rabbit polyclonal) used were PKCα (sc-208), -βI (sc-209), -δ (sc-213), and -ε (sc-214) from Santa Cruz Biotechnology (Santa Cruz, CA). PKCε was from Cell Signaling Technology (Beverly, MA) and phospho-MARCKS was from Proteintech Group (Chicago, IL). Anti-rabbit IgG-HRP secondary antibody was from Cell Signaling Technology.

Data analysis. Data analysis. Analysis of variance was used to evaluate the significance of intergroup differences in the permeability studies. A value of $P < 0.05$ was considered significant for the comparisons.

RESULTS

Pharmacological inhibition of PMA-induced hyperpermeability across RLMEC monolayer. The phorbol ester PMA is known to activate both cPKC and nPKC isoforms. Furthermore, activation of PKC has been shown to result in endothelial barrier dysfunction (11, 27). RLMEC were treated with increasing concentrations of PMA for 15 min and assessed for albumin flux across the monolayer. As shown in Fig. 1, a significant permeability increase occurs at 10 nM PMA and continues to increase up to 1 μM. At concentrations above 1 μM, PMA elicits a toxic effect on the Transwell membrane-grown monolayer as seen by cell detachment. To determine which PKC isoform(s) is responsible for PMA-induced hyperpermeability, four different pharmacological inhibitors were employed in combination with PMA. None of these inhibitors had a significant effect on basal permeability (Fig. 2A). As expected, BIM, a general PKC inhibitor at 10 nM, blocked the permeability increase elicited by PMA (Fig. 2B). Rottlerin, which selectively inhibits PKCβ at 5 μM, and G06983, which inhibits all PKC isoforms but not PKD at 1 μM, both significantly attenuated PMA-induced hyperpermeability (Fig. 2B).

However, PMA-induced permeability increases were not attenuated by pretreatment with G06976, a PKCα and PKCβI inhibitor at 10 nM (Fig. 2B). These results suggest that it is the

Table 1. Sequences of antisense oligonucleotides

<table>
<thead>
<tr>
<th>Oligonucleotide</th>
<th>Sequence (5’-3’)</th>
<th>Target Region on PKC mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>AAAACCTGACGCCAGT</td>
<td>−1to+14</td>
</tr>
<tr>
<td>βI</td>
<td>TCGCCATGGCCGGCCG</td>
<td>−10to+8</td>
</tr>
<tr>
<td>δ</td>
<td>GTGCCATGGAGAAGCTT</td>
<td>−13to+7</td>
</tr>
<tr>
<td>ε</td>
<td>TTGAAGTACACATGG</td>
<td>−1to+14</td>
</tr>
<tr>
<td>PKD</td>
<td>GACGCGAGGCGCTGTCG</td>
<td>−3to+18</td>
</tr>
</tbody>
</table>

Sequences of antisense oligonucleotides (GAACGCGAGGCGCTGTCG) were from Sigma.

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novel PKC isoform δ and possibly others that are playing prominent roles in PMA-induced hyperpermeability.

Antisense oligonucleotide depletion of PKC isoforms. To make a more specific evaluation of which PKC isoforms are important to PMA-induced activity in microvascular endothelial cells, isoform-specific antisense oligonucleotides were designed against PKCα, -βI, -δ, -ε, and PKD. It should be pointed out that we were not able to detect PKCβII, -γ, -η, or -θ in the RLMEC. As shown in Fig. 3, antisense treatment was successful in depleting the cells of most, if not all, of the five isoforms we were able to detect in untreated cells. To confirm the specificity of the oligonucleotides, lysate was analyzed for both the isoform which antisense treatment was intended to deplete and two of the other isoforms. In every case, the antisense oligonucleotide was effective against its specific isoform and had no effect on expression of the others (Fig. 3).

PMA-induced hyperpermeability across RLMEC monolayers after antisense oligonucleotide treatment. Now that we had the means to specifically deplete the cells of individual PKC isoforms, we sought to determine which isoform(s) was responsible for PMA-induced hyperpermeability. The antisense oligonucleotides had no significant effects on basal permeability (Fig. 4A). Figure 4B shows that treatment with antisense oligonucleotides directed against PKCα, -βI, and -ε did not result in attenuation of the permeability increases elicited by PMA. However, when cells were depleted of PKCδ or PKD before PMA treatment, hyperpermeability responses were blocked (Fig. 4B).

PMA-induced MARCKS phosphorylation. Having deduced which PKC isoforms elicit hyperpermeability responses, we sought to evaluate the actual PKC activity in the cells after PMA treatment. Phosphorylation of MARCKS protein is a widely used indicator of PKC activity in the cell. Both conventional and novel PKC isoforms are known to phosphorylate MARCKS (5, 25). Treatment of RLMEC with increasing concentrations of PMA led to dosage-dependent increases in MARCKS phosphorylation (Fig. 5), reaching maximal levels at 1 μM. To determine which PKC isoform(s) is responsible for PMA-induced MARCKS phosphorylation, we again employed antisense oligonucleotides. PMA-induced MARCKS phosphorylation occurred rapidly (5 min) and stayed at a level approximately fourfold above basal levels for 60 min (Fig. 6). We found that depletion of PKCδ or PKD before PMA treatment resulted in attenuation of MARCKS protein phosphorylation, i.e., PKC activity (Fig. 6). Furthermore, PKCα, -βI, and -ε depletion had little, if any, effect on PMA-induced PKC activity (Fig. 6).

PMA-induced PKC isoform translocation. We employed another method to evaluate activity levels of the PKC isoforms of interest, cytosol vs. membrane distribution. Under basal conditions, PKCα, -βI, -δ, -ε, and PKD exist for the most part in the cytosolic fraction (Fig. 7). After PMA treatment for 5–60 min, we saw incomplete translocation of PKCα, -βI, and -ε from the cytosol to the membrane (Fig. 7). However, the translocation of PKCδ and PKD to the membrane fraction after PMA treatment was complete at 15 and 30 min, with an apparent initial return to the cytosol at 60 min (Fig. 7). This finding further corroborates activity levels measured by MARCKS phosphorylation, as well as the permeability studies implicating PKCδ and PKD as the major kinases responsible for cellular activity after phorbol ester treatment.
DAG-induced hyperpermeability and MARCKS phosphorylation. Although PMA is a widely accepted and utilized PKC activator, we sought to determine which PKC isoforms are responsible for DAG (a natural PKC activator)-induced cellular responses. As shown in Fig. 8, the effect of a 15-min DAG treatment on monolayer hyperpermeability was maximal at 10 μM and resulted in a 38% increase in albumin flux. Furthermore, depletion of PKCζ and PKD significantly attenuated the DAG-induced hyperpermeability responses, whereas depletion of PKCε, -α, and -δ did not (Fig. 8). To further measure the effect of DAG on cellular responses, we looked at MARCKS phosphorylation and found that maximal phosphorylation was also reached with 10 μM DAG treatment (Fig. 8). In addition, DAG-induced MARCKS phosphorylation was lowered to near basal levels upon depletion of PKCζ and PKD. It should also be pointed out that depletion of PKCα apparently reduced the effects of DAG on hyperpermeability and MARCKS phosphorylation (Fig. 8). Although these reductions were not statistically significantly, the results suggest that PKCα may play a role in DAG-induced cellular responses.

DISCUSSION

PKC is known to play an important role in the signaling mechanisms of many different cell types (16, 19, 26, 30). Specifically, the role of PKC in the mediation of microvascular hyperpermeability has been widely established. Activation and inhibition of PKC generally results in increases and decreases, respectively, in permeability (1, 6, 11, 17). We found that both PMA and DAG induced hyperpermeability across RLMEC monolayers in a dosage-dependent manner. Furthermore, PMA and DAG showed the same effects on MARCKS phosphorylation, an accepted measure of PKC activity. However, little was known with regard to which PKC isoforms are involved in the regulation of microvascular endothelial cell barrier function. This study, for the first time, implicates two isoforms required for barrier dysfunction in pulmonary microvascular endothelium, namely PKCζ and PKD.

Fig. 3. Antisense depletion of PKC isoforms and PKD. RLMEC were grown to confluence on 60-mm dishes and depleted of PKCα, -ε, -α, -ζ, -δ, or PKD by treating with antisense phosphorothioate oligonucleotides for 48 h as described in MATERIALS AND METHODS. After lysis, 10 μg of cellular protein were subjected to Western analysis for total PKC isoform content. Antibodies for immunoblotting were PKCα (A), PKCζ (B), PKCζ (C), PKCε (D), and PKD (E). Each panel depicts control cell lysate and 3 different antisense treatment lysates to show the inhibition specificity of each oligonucleotide. The experiments were repeated 3 times, and representative Westerns are shown.

Fig. 4. Effects of PKC isoform-specific antisense oligonucleotides on PMA-induced monolayer permeability. RLMEC were grown to confluence on Transwell membranes and transfected with antisense oligonucleotides for 48 h as described in MATERIALS AND METHODS. The effects of antisense oligonucleotides on basal permeability are shown in A. In B, transfected cells were treated with PMA (1 μM) for 15 min before permeability measurements. Pa was calculated after 30 min as described in MATERIALS AND METHODS. Pa values are expressed as percentages of basal level. For each treatment, n = 5. *P < 0.05 vs. basal; #P < 0.05 vs. PMA.

Fig. 5. PMA-induced myristoylated alanine-rich C kinase substrate (MARCKS) phosphorylation. RLMEC were grown to confluence on 60-mm dishes and treated with increasing concentrations of PMA for 15 min. After lysis, 10 μg of total protein were subjected to Western analysis for detection of phosphorylated MARCKS. The experiments were repeated 3 times, and a representative Western is shown.
We were able to detect four PKC isoforms (α/H9251, βI/H9252, δ/H9254, and ε/H9280) and PKD by Western blot from RLMEC lysate. Several parameters were employed to assess the activity conferred by the various kinases. One such measure of activity, cytosol-to-membrane translocation, showed complete movement of PKCδ/H9254 and PKD to the membrane fraction upon PMA stimulation. PKCα/H9251 and PKCβI/H9252 partially relocated from cytosol to membrane, whereas PKCε was found in approximately equal amounts in the two fractions. These findings coincide with the pharmacological inhibitor analysis, suggesting that it was the novel, not conventional, isoforms responsible for PMA-induced PKC activity and hyperpermeability responses. However, the specificity of pharmacological inhibitors is often called into question, and a more precise technique for determining the role of individual isoforms was sought.

By specifically inhibiting individual isoforms employing antisense technology, we determined that PKCδ/H9254 and PKD were both required for PMA- and DAG-induced hyperpermeability responses across the endothelial monolayer. In addition, depletion of either of these isoforms attenuated PMA- and DAG-induced MARCKS phosphorylation. Antisense oligonucleotides directed at PKCα/H9251 and PKCβI showed little or no effect on PMA-induced hyperpermeability or MARCKS phosphorylation. However, we saw an apparent reduction in DAG-induced hyperpermeability and MARCKS phosphorylation upon depletion of PKCε. This suggests that perhaps PKCδ plays a role in maximizing DAG-induced hyperpermeability in the microvascular endothelium but is not required for a response, as in the case with PKCδ and PKD. These findings suggest that PKCδ and PKD are either essential components of a single pathway or perhaps involved in different pathways, both of which participate in the hyperpermeability response. Recent work, which has uncovered apparent PKC kinase cascades, suggests the former. Studies from two independent groups have discovered a link between PKCδ and PKD. First, in experiments on COS-7 cells, evidence of a direct PKCδ/PKD phosphorylation cascade in which PKD is phosphorylated at Ser744 and Ser748 of its activation loop was shown (28).
Second, PKCε has been identified as an upstream kinase for PKD in the regulation of downstream effectors (3). Further evidence of PKC signaling cascades is demonstrated by the discovery that PKCζ both colocalizes with and activates PKD via phosphorylation of PKDSer1738/742 (4). Most importantly with regard to our work, a recent study has shown that upon thrombin activation of aortic smooth muscle cells, PKD activation is mediated in a PKCζ-dependent fashion (21). Our study is further evidence of the involvement and possible interaction between PKD and PKCζ, specifically upon activation by phorbol ester. These findings illustrate the newly discovered complexities in PKC signaling cascades, and our work is the first to directly implicate the two kinases with both PKC activity and cellular abnormalities, i.e., hyperpermeability, in the microvascular endothelium.

As mentioned above, there was incomplete translocation from the cytosol to the membrane with the PKC isoforms α, βι, and ε upon PMA stimulation of the cells. However, we found these isoforms to be dispensable with regards to PMA- and DAG-induced PKC activity as measured by MARCKS phosphorylation and hyperpermeability responses in this cell line. It could be that in the pulmonary microvasculature, specific activation by PKC or DAG is propagated mainly by PKCζ and PKD, whereas the other isoforms play something of a supportive role. In other words, partial activation of PKCζ, -βι, and -ε may itself induce downstream activation of PKCζ and PKD. Depletion of PKCζ, -βι, or -ε individually may in itself not be sufficient to significantly attenuate PMA- and DAG-induced responses. That is to say, the other two kinases may be able to continue the signaling cascade in the absence of the other. The two studies mentioned above (3, 28) that discovered a link between PKCα and PKD support this line of reasoning. Perhaps PKCζ and -βι play a similar role to PKCζ in subsequent regulation of PKCζ and PKD. Although there is redundancy in the signaling cascade upstream of PKCζ and PKD, the continued propagation of PKC activity with respect to hyperpermeability is dependent on both of these enzymes. In summary, our work identifies PKCζ and PKD as being required for PMA- and DAG-induced PKC activity and hyperpermeability responses in pulmonary microvascular endothelial cells.

GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants HL-61507 and HL-70752 (to S. Y. Yuan) and a Department of Veterans Affairs Veterans Integrated Service Network 17 Grant (to J. H. Tinsley).

REFERENCES


