Platelet-conditioned medium increases endothelial electrical resistance independently of cAMP/PKA and cGMP/PKG

JONATHAN P. GAINOR, CHRISTINE A. MORTON, JARED T. ROBERTS, PETER A. VINCENT, AND FRED L. MINNEAR
Center for Cardiovascular Sciences, Albany Medical College, Albany, New York 12208-3479
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Gainor, Jonathan P., Christine A. Morton, Jared T. Roberts, Peter A. Vincent, and Fred L. Minnear. Platelet-conditioned medium increases endothelial electrical resistance independently of cAMP/PKA and cGMP/PKG. Am J Physiol Heart Circ Physiol 281: H1992–H2001, 2001.—Platelets release a soluble factor into blood and conditioned medium (PCM) that decreases vascular endothelial permeability. The objective of this study was to determine the signal-transduction pathway that elicits this decrease in permeability. Permeability-decreasing activity of PCM was assessed by the real-time measurement of electrical resistance across cell monolayers derived from bovine pulmonary arteries and microvessels. Using a desensitization protocol with cAMP/protein kinase A (PKA)-enhancing agents and pharmacological inhibitors, we determined that the activity of PCM is independent of PKA and PKG. Genistein, an inhibitor of tyrosine kinases, prevented the increase in endothelial electrical resistance. Because lysophosphatidic acid (LPA) has been proposed to be responsible for this activity of PCM and is known to activate the G protein, inhibitors of the G protein pertussis Cxin and of the associated phosphatidylinositol 3-kinase (PI3K) wortmannin were used. Pertussis toxin and wortmannin caused a 10- to 15-min delay in the characteristic rise in electrical resistance induced by PCM. Inhibition of phosphorylation of extracellular signal-regulated kinase with the mitogen-activated kinase kinase inhibitors PD-98059 and U-0126 did not prevent the activity of PCM. Similar findings with regard to the cAMP protocols and inhibition of G<sub>i</sub> and PI3K were obtained for 1-oleoyl-LPA. These results demonstrate that PCM increases endothelial electrical resistance in vitro via a novel, signal transduction pathway independent of cAMP/PKA and cGMP/PKG. Furthermore, PCM rapidly activates a signaling pathway involving tyrosine phosphorylation, the G<sub>i</sub> protein, and PI3K.

PERMEABILITY: signal transduction; G<sub>i</sub> protein; phosphatidylinositol 3-kinase; extracellular signal-regulated kinase; tyrosine kinase; lysophosphatidic acid

PLATELETS SUPPORT THE FUNCTION of the vascular endothelium as a semipermeable barrier to the passage of water and protein. In patients with low platelet counts (thrombocytopenia), petechial and purpuric hemorrhages and edema develop in the skin and mucus membranes (6, 13). In animals, thrombocytopenia has been shown to increase protein permeability in the vasculatures of the lung (18), ear (3), thyroid (8), and heart (19). Repletion with platelet-rich plasma reverses these abnormalities in clinical and experimental settings (3, 8, 18, 19). In vitro experimentation has demonstrated that the addition of platelets or platelet-conditioned medium (PCM) to endothelial cell monolayers decreases protein permeability (2, 10, 30, 31, 34). Although much effort has been focused on identifying the active factors, little is known about the cell-signaling pathway that initiates the permeability-decreasing activity of platelets.

A plethora of literature (4, 14, 20–22, 28, 39) has documented the ability of the cAMP/protein kinase A (PKA) signal-transduction pathway to decrease water and protein transport across the vascular endothelium. The cGMP/protein kinase G (PKG) pathway has also been implicated in tightening the endothelial barrier (12). Therefore, the first objective of the present study was to determine whether platelets decrease endothelial permeability via cAMP/PKA and cGMP/PKG. Two approaches, a desensitization protocol with cAMP-enhancing agents and pharmacological inhibition of PKA or PKG, were taken to determine the importance of the cAMP/PKA- and cGMP/PKG-signaling pathways.

Lysophosphatidic acid (LPA) has been proposed as the active factor responsible for the permeability-decreasing activity of platelets (2). In neurites and fibroblasts, LPA is known to initiate cell signaling via activation of the G proteins, inhibitory (G<sub>i</sub>), G<sub>q</sub>, and G<sub>12/13</sub> (9, 23, 24, 37). Therefore, we also focused on the G<sub>i</sub> protein signaling pathway, which can influence the levels of intracellular cAMP, to determine whether tyrosine phosphorylation and specific proteins involved in this pathway, G<sub>i</sub>, phosphatidylinositol 3-kinase (PI3K), and the extracellular signal-regulated kinase (ERK), initiate the activity of PCM as well as LPA.

METHODS

Materials. Bovine endothelial cells derived from pulmonary arteries and pulmonary microvessels were obtained from Vec Technologies, fetal bovine serum was from Summit Biotechnologies, and gentamicin sulfate was from Bioproducts. KT-5720 and H-89 were from Alexis, KT-5823, pertussis

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sin toxin, wortmannin, LY-294002, PD-98059, and U-0126 were from Biomol, genistein was from Calbiochem, and 1-oleoyl-LPA was from Avanti Polar Lipids. Electric cell-substrate impedance sensing (ECIS) apparatus and gold-coated electrodes were from Applied Biophysics; SDS-PAGE mini-slab gel was from Bio-Rad; polyclonal antibody specific for phosphorylated ERK (p44/p42, thr 204/tyr 202) was from New England BioLabs; and enhanced chemiluminescence kit was from American International. All other compounds and materials were purchased from Sigma.

**Platelet isolation and preparation of PCM.** Platelets were isolated from fresh nonirradiated platelet packs purchased from the local blood bank. Isolated platelets were brought to a concentration of $2 \times 10^9$ platelets/ml in modified (Ca$^{2+}$/Mg$^{2+}$ free) Tyrode buffer (in M) 0.137 NaCl, 0.003 KCl, 0.012 NaHCO$_3$, 0.006 glucose, and 0.004 Na$_2$HPO$_4$, pH 7.4, incubated in plastic round-bottom tubes for 2 h, and centrifuged at 600 $g$ for 20 min. The supernatant was designated as PCM and aliquoted and stored at 4°C for immediate use or at −80°C for later use.

**Endothelial cell culture.** Endothelial cells from bovine pulmonary arteries (passages 3–20) and microvessels (passages 3–20) were grown in MCDB-131, 10% fetal bovine serum, and 50 $\mu$g/ml of gentamicin sulfate. For the measurement of endothelial electrical resistance using ECIS, cells were seeded onto 1% gelatin-coated gold electrodes (60,000–100,000 cells onto 0.5 cm$^2$ wells). Porcine dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), cells were seeded onto 60-mm petri dishes (500,000 cells/dish). Cells were grown to confluence (3–4 days) in a humidified environment maintained at 37°C and 5% CO$_2$. Before commencement of an experiment, cell culture medium was changed from 10% serum to serum-free MCDB-131, and the cells were placed in the ECIS incubator and allowed to equilibrate for 2 h.

**Assessment of endothelial permeability.** Continuous measurement of electrical resistance across endothelial cell monolayers was used to determine changes in endothelial permeability. Electrical resistance was measured by using the methodology known as ECIS (7, 28, 30, 35). The basic setup to measure endothelial electrical resistance is shown in Fig. 1. Endothelial cells were grown to confluence on small gold electrodes ($5 \times 10^{-4}$ cm$^2$) in culture medium, which functioned as the electrolyte. The small gold electrode, covered by confluent endothelial cells, and a larger gold counter electrode ($-2$ cm$^2$) were connected to a phase-sensitive, lock-in amplifier. A 1-V, 4000-Hz alternating current was passed through a 1-M$\Omega$ resistor to approximate a constant current source of 1 $\mu$A. Treating the cell-electrode system as a simple series resistance-capacitance circuit, the measured changes in the in-phase voltage and the out-of-phase voltage can be used to calculate these values for resistance and capacitance. Voltage and phase data were stored and processed with the use of a personal computer. The same computer controlled the output of the amplifier and switched the measurements to different electrodes in each of two, eight-well arrays during the course of an experiment. The small size of the cell-seeded electrode is the critical feature of the system. When electrodes of $10^{-3}$ cm$^2$ or smaller are used, the impedance at the small electrode dominates the system, allowing this impedance to be measured and also allowing for assessment of cellular morphology. The measurement of electrical impedance was obtained in real time every minute before and for 30–60 min after treatment of the endothelial cells and reported as the resistive portion of electrical imped ance.

**Measurement of the phosphorylation levels of p44/p42 ERK.** Endothelial cells were pretreated for 1 h with 10 or 30 $\mu$M PD-98059 or with 10 $\mu$M U-0126, then treated with PCM. At 10 min, cells were placed on ice, washed with phosphate-buffered saline, and treated with 100 $\mu$l of SDS sample buffer with dithiothreitol (62.5 mM Tris·HCl, 2% wt/vol SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% wt/vol bromphenol blue) to lyse the cells. Endothelial cells were scraped from the petri dishes with a rubber policeman, and cell lysates were transferred to a microcentrifuge tube. Samples were boiled for 5 min to denature proteins. Samples (20 $\mu$l) were loaded onto each stacker and run on a 10% SDS-PAGE mini-slab gel according to the method of Laemmli (15), and then transferred to a nitrocellulose membrane. Membranes were incubated with a polyclonal antibody to phosphorylated ERK (p44/p42, thr 204/tyr 202). Enhanced chemiluminescence was used for antibody detection. Phosphorylation levels of p44/p42 ERK were quantified by densitometry and normalized to control conditions.

**Experimental drugs.** PCM was administered to endothelial cell monolayers as a 1:4 dilution of the stock concentration ($2 \times 10^9$ platelets/ml) of PCM. LPA was dissolved in a Ca$^{2+}$-free phosphate-buffered saline solution to yield a stock concentration of 500 $\mu$m and diluted further in serum-free MCDB-131. Isoproterenol and 8-bromo-CAMP were dissolved in MCDB-131. Pertussis toxin was reconstituted in sterile water and diluted in MCDB-131; the vehicle control was sterile water with 10 mM NaP and 50 mM NaCl. Forskolin, KT-5720, H-89, KT-5823, genistein, wortmannin, LY-294002, and U-0126 were dissolved initially in 100% dimethyl sulfoxide (DMSO) to yield a stock concentration and diluted further in serum-free MCDB-131. PD-98059 was also
dissolved initially in 100% DMSO, but the primary dilution was made in MCDB-131 containing 5% fetal bovine serum.

**Statistics.** Each study that measured endothelial electrical resistance consisted of at least five different experiments. Western blots were analyzed quantitatively from at least three separate experiments. Data were analyzed using a two-way analysis of variance with repeated measures (38). Differences between treatment groups from the control group were analyzed with the Newman-Keuls post hoc test. Statistical significance was set at $P < 0.05$.

**RESULTS**

**PCM functions via a cAMP/PKA-independent signaling pathway.** A desensitization protocol was used first to determine whether PCM decreases endothelial permeability via cAMP/PKA. Isoproterenol (1 μM), forskolin (10 μM), and 8-bromo-cAMP (1 mM) were incubated individually with endothelial cell monolayers as two separate challenges that were 45 min apart. Cells responded to the first challenge of each of these agents with an increase in electrical resistance (Fig. 2). After the second challenge with each agent, electrical resistance did not increase, indicative of desensitization. In contrast, desensitization was not observed when PCM was administered as the second challenge (Fig. 2). Similar responses were obtained with 2 and 5 μM isoproterenol, respectively (data not shown). When the challenges were reversed, PCM administered as the first treatment desensitized the increase in endothelial electrical resistance induced by the second challenge of isoproterenol, 8-bromo-cAMP, and PCM (Fig. 3).

In a second protocol, PKA was inhibited with 2 μM KT-5720 or 10 μM H-89. KT-5720, as well as H-89 (data not shown), blocked the increased endothelial electrical resistance induced by 8-bromo-cAMP (Fig. 4A). Similarly, KT-5720 blocked the increased endothelial electrical resistance induced by isoproterenol and forskolin (data not shown). In contrast, these two PKA inhibitors did not block the increased endothelial electrical resistance induced by PCM (Fig. 4B).

**PCM does not function via PKG.** To determine whether PKG was responsible for the increased electrical resistance induced by PCM, the endothelial cell monolayers were pretreated for 1 h with 5 or 10 μM KT-5823, an inhibitor of PKG, and were then administered PCM or 1 mM 8-bromo-cGMP, a cell-permeable analogue of cGMP. KT-5823 blocked the increase in endothelial electrical resistance induced by 8-bromo-cGMP (Fig. 5A) but did not inhibit the increased electrical resistance induced by PCM (Fig. 5B).

**Inhibitor of tyrosine kinase activity prevents PCM activity.** There are a number of steps in cell signaling that require tyrosine phosphorylation. Pretreatment with the tyrosine kinase inhibitor genistein for 1 h at 50 and 100 μM prevented the increase in endothelial electrical resistance induced by PCM (Fig. 6).

**Inhibitors of Gi and PI3K delay PCM activity.** The recently proposed active platelet factor LPA (2) has been shown to activate the G$_i$ pathway, which is pertussis toxin sensitive (24, 37) and involves PI3K (9, 11, 23, 32). To determine whether the PCM-induced in-

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**Fig. 2.** cAMP-enhancing agents do not desensitize permeability-decreasing activity of platelet-conditioned medium (PCM). Electrical resistance experimental/baseline (E/B) across endothelial cell monolayers derived from pulmonary microvessels increased on treatment at 0 min (arrow) with isoproterenol (Iso; A), forskolin (B), and 8-bromo-cAMP (C). A second treatment 45 min later (arrow) with the same agents (open symbols) resulted in no change in electrical resistance. In contrast, electrical resistance increased when PCM (closed symbols) was administered as the second treatment. Values are means ± SE; $n = 6$ cell monolayers per group. *$P < 0.05$, compared with corresponding time point in open symbol groups and for all time points to the right.
crease in endothelial electrical resistance is initiated by a pertussis-toxin-sensitive \( G_i \) protein, endothelial cell monolayers were pretreated for 3 h with 50 and 100 ng/ml of pertussis toxin, and were then treated with PCM. Pertussis toxin blocked the characteristic rapid rise in endothelial electrical resistance induced by PCM (Fig. 7A). Electrical resistance eventually increased to a similar level as that induced by PCM but the increase was delayed for 10 to 15 min. Similar results were obtained by using two mechanistically different inhibitors of PI3K, wortmannin, and LY-294002. At doses that have been reported to be specific for PI3K, wortmannin (15 and 30 nM; Fig. 7B) and LY-294002 (0.5 and 1 \( \mu \)M; data not shown) also

Fig. 4. Inhibitor of protein kinase A (PKA) does not prevent activity of PCM. cAMP (A, closed symbols) and PCM (B, closed symbols) increased electrical resistance across endothelial cell monolayers derived from pulmonary arteries. Pretreatment of endothelial cell monolayers for 1 h with KT-5720, inhibitor of PKA, prevented the increased electrical resistance induced by addition of cAMP at 0 min (A, open symbols). In contrast, KT-5720 did not block the increased electrical resistance induced by PCM (B, open symbols). Values are means \( \pm \) SE; \( n = 6 \) cell monolayers per group. *\( P < 0.05 \), compared with corresponding time point in dimethyl sulfoxide (DMSO) group (closed symbols) and for all time points to the right. DMSO, vehicle for KT-5720.

Fig. 3. PCM attenuates the permeability-decreasing activity of Iso and cAMP. Electrical resistance across endothelial cell monolayers derived from pulmonary microvessels increased on treatment at 0 min (arrow) with PCM (closed symbols in A, B, and C). This first treatment with PCM significantly attenuated the second treatment 45 min later (arrow) with Iso (A), cAMP (C), and PCM. All three agents elevated endothelial electrical resistance after pretreatment with control vehicle MCDB-131 (open symbols). Values are means \( \pm \) SE; \( n = 6 \) cell monolayers per group. *\( P < 0.05 \), compared with corresponding time point in open symbol groups from 0 to 45 min; \( ^{t}P < 0.05 \), compared with 45-min time point in open symbol groups.
caused a delay of 10 to 15 min in the initial, rapid rise in endothelial electrical resistance induced by PCM. Inhibition of ERK phosphorylation does not prevent PCM activity. To determine the involvement of ERK, a primary kinase activated by the Gt signaling pathway, the upstream kinase mitogen-activated protein kinase kinase (MEK) was inhibited with PD-98059 (10 and 30 μM) or U-0126 (10 μM). Pretreatment for 1 h with 10 or 30 μM PD-98059 (Fig. 8A) or 10 μM U-0126 (Fig. 8B) had no effect on PCM activity, although there was a 3-min delay in the onset of PCM activity in the presence of U-0126. However, these doses of PD-98059 and U-0126 significantly inhibited the increased phosphorylation of ERK in PCM-treated cells (Fig. 8, C and D). The lack of a correlation between the effects of MEK inhibition on endothelial electrical resistance and ERK phosphorylation demonstrated that PCM does not decrease endothelial permeability via activation of ERK.

LPA also functions independently of cAMP and is affected by inhibitors of Gt and PI3K. Because LPA may be the active factor in PCM and is known to activate the Gt protein, we repeated the experiments involving cAMP, Gt, and PI3K using 1-oleoyl-LPA (10 μM). The desensitization protocol with the cAMP-enhancing agents and the PKA inhibitor, KT-5720, produced similar responses with LPA as occurred with PCM (Fig. 9). Whereas the two challenges of 8-bromo-cAMP resulted in a desensitization response, LPA was not inhibited by the prior challenge of 2 mM 8-bromo-cAMP. Similarly, 2 μM KT-5720 prevented the 8-bromo-cAMP response but did not prevent the rapid increase in endothelial electrical resistance induced by LPA. KT-5720 did attenuate the maximum increase in electrical resistance after LPA treatment; however, this attenuation may have been influenced by the two pretreatments, DMSO and KT-5720 with DMSO. Electrical resistance decreased initially due to the DMSO vehicle, then rebounded back to a higher level in the KT-5720 group than in the vehicle DMSO group, which would affect the calculation of the normalized experimental-to-baseline values.

Slightly different results were obtained using the inhibitors of the Gt protein and PI3K (Fig. 9). Similar to the response for PCM, the PI3K inhibitor, LY-294002 (0.5 μM) also caused a delay in the activity of LPA. However, 100 ng/ml of pertussis toxin prevented the activity of LPA, compared with delaying the activity of PCM.

DISCUSSION

The objective of the present study was to determine the signal-transduction pathway that PCM activates to decrease vascular endothelial permeability. Perme-
ability was assessed by the real-time measurement of electrical resistance across endothelial cell monolayers. Desensitization with three cAMP/PKA-enhancing agents and inhibitors of PKA and PKG did not prevent the increase in endothelial electrical resistance induced by PCM. Inhibition of ERK activity also had no effect on the activity of PCM. Instead, an inhibitor of tyrosine kinases prevented PCM activity, and inhibitors of the G_i protein and PI3K delayed by 10 to 15 min the characteristic rise in endothelial electrical resistance induced by addition of PCM at 0 min. When pulmonary artery endothelial cells were pretreated for 3 h with pertussis toxin (PTx), inhibitor of G_i protein, a 10- to 15-min delay was observed in the characteristic rise in electrical resistance induced by addition of PCM at 0 min. Inhibitors of PI3K, then treated with PCM at 0 min. Values are means ± SE; n = 6 cell monolayers per group for A and 5 for B. *P < 0.05 compared with corresponding time point in PCM groups. DMSO, vehicle for wortmannin.

Fig. 7. Inhibitors of inhibitory G protein (G_i) and phosphatidylinositol 3-kinase (PI3K) delay activity of PCM. A: pretreatment of pulmonary artery endothelial cells for 3 h with pertussis toxin (PTx), inhibitor of G_i protein, caused a 10- to 15-min delay in the characteristic rise in endothelial electrical resistance induced by addition of PCM at 0 min. B: similar results were obtained when pulmonary artery endothelial cells were pretreated for 20 min with wortmannin, inhibitor of PI3K, then treated with PCM at 0 min. Values are means ± SE; n = 6 cell monolayers per group for A and 5 for B. *P < 0.05 compared with corresponding time point in PCM groups. DMSO, vehicle for wortmannin.

except that the inhibitor of the G_i protein, pertussis toxin, did not cause a delay in activity but instead prevented the activity of LPA.

Permeability across endothelial cells grown as a monolayer was assessed with a methodology known as ECIS for electric cell-substrate impedance sensing. This methodology was developed to study the dynamic behavior of cells in culture (7, 35). ECIS measures in real-time the changes in impedance of an endothelial cell monolayer to a weak, noninvasive, alternating current signal. We have demonstrated that measurements of increases and decreases in electrical resistance correlate with decreases and increases, respectively, in 125I-labeled albumin clearance and in microscopic gaps across endothelial cell monolayers (28, 30). The increase in endothelial electrical resistance induced by PCM in this study also correlates with the PCM induced decrease in permeability of sodium fluorescein (mol wt 342) across endothelial cell monolayers derived from bovine aorta (2, 10).

Thrombocytopenia increases vascular permeability, resulting in petechial and purpuric hemorrhages in the skin and tissue edema (3, 8, 18, 19). Repletion with platelet-rich plasma improves these conditions (3, 8, 18, 19). This permeability-decreasing activity of platelets appears to reside in a releasable, soluble factor because PCM consistently increases electrical resistance and decreases albumin permeability across endothelial cell monolayers derived from bovine aorta, pulmonary arteries, and pulmonary microvessels (2, 10, 30, 31, 34). Many soluble factors have been proposed, studied, and rejected as the active factor released from platelets. These factors include serotonin, norepinephrine, cyclooxygenase products, adenosine, adenine nucleotides, and a protein (30, 31, 34). The most recently proposed active platelet factor is a phospholipid LPA (2).

Although much effort has been generated to identify this soluble platelet factor, there is a paucity of information concerning the cellular mechanisms by which the active platelet factor functions to decrease endothelial permeability. Two signal-transduction pathways have been studied in depth with regards to endothelial permeability. The cAMP/PKA-signaling pathway is well known to prevent and reverse an increase in vascular permeability induced by a variety of mediators, diseases, and syndromes (4, 14, 20–22, 28, 39). The effect of the cGMP/PKG-signaling pathway on endothelial permeability, however, is controversial and may be cell, tissue, and/or organ specific (12). When we considered the long list of publications involving the above two signaling pathways, we initially asked the question whether one of these two pathways is responsible for the permeability-decreasing activity of PCM. Two approaches were taken: the first used a desensitization protocol with three different cAMP/PKA-enhancing agents and the second used inhibitors of PKA and PKG. The first challenge with the use of isoproterenol (a β_2-adrenergic receptor agonist), forskolin (a cell-permeable activator of adenylate cyclase), or 8-bromo-cAMP (a cell-permeable analogue of cAMP) in-
creased endothelial electrical resistance with an activity profile similar to that of PCM. This first challenge prevented (desensitized) the change in electrical resistance when the second challenge (45 min apart) with the same agent was administered. In contrast, these three cAMP/PKA-enhancing agents did not prevent the activity of PCM when PCM was administered as the second challenge. The inhibitors of PKA (KT-5720 and H-89) and of PKG (KT-5823) blocked their respective kinase activators, isoproterenol, forskolin, 8-bromo-cAMP, and 8-bromo-cGMP but had no effect on the activity of PCM. Furthermore, 8-bromo-cGMP consistently initiated a slower increase in endothelial electrical resistance compared with PCM. We conclude from these studies that PCM increases endothelial electrical resistance via a cellular mechanism independent of PKA and PKG.

Interestingly, when the challenges were reversed in the desensitization protocol, PCM significantly attenuated the increased endothelial electrical resistance induced by isoproterenol and 8-bromo-cAMP. This desensitization of the isoproterenol response could result from activation of the G\textsubscript{i} protein by PCM and down-regulation of the cAMP pathway. The mechanism for desensitization of the 8-bromo-cAMP response is less clear. It is possible that the active factors in PCM and cAMP/PKA activate parallel pathways that converge on common final targets that mediate the increase in endothelial electrical resistance, our assessment of endothelial barrier function. According to the data in Figs. 2 and 3, activation of the signal transduction pathway that is independent of cAMP would have a greater effect on modulating endothelial barrier function.

We next determined whether PCM might influence endothelial permeability via the G\textsubscript{i}-ERK-signaling pathway. The recently proposed active platelet factor LPA (2) is known to stimulate signal-transduction pathways initiated by G\textsubscript{i}, G\textsubscript{q}, and G\textsubscript{12/13} protein-coupled receptors (9, 23, 24). We focused initially on the G\textsubscript{i}-ERK-signaling cascade. We recently proposed active platelet factor as an early mediator in the suspected pathway (9, 23); MEK, the upstream kinase of ERK, is a dual specific kinase that causes phosphorylation of the tyrosine phosphorylation of dynamin (1). Genistein, an inhibitor of the activity of tyrosine kinases, prevented PCM activity. There are a number of steps in the G\textsubscript{i}-ERK signaling pathway that require tyrosine phosphorylation. A tyrosine kinase has been proposed as an early mediator in the suspected pathway (9, 23); MEK, the upstream kinase of ERK, is a dual specific kinase that causes phosphorylation of ERK at tyrosines and threonines; assembly of the protein scaffold involved in Ras activation is dependent on tyrosine phosphorylation; and internalization of the G-protein-coupled receptor complex, which may be important in activation of downstream kinases, is dependent on the tyrosine phosphorylation of dynamin (1).

Inhibitors of the G\textsubscript{i} protein (pertussis toxin) and of PI3K (wortmannin and LY-294002) resulted in a 10–15 min delay in the characteristic rapid rise in endothelial electrical resistance induced by PCM. These findings indicate that tyrosine phosphorylation and both the G\textsubscript{i} protein and PI3K are involved in the PCM-signaling cascade. Because the inhibitors of G\textsubscript{i} and PI3K only delayed the increase in electrical resistance, other G proteins and signal-transduction pathways could be involved in the cellular response and/or the active platelet factor may internalize into the cell.
in some way and bypass upstream signaling proteins. One possible explanation is that the active platelet factor initiates a fast signal-transduction pathway involving Gi and PI3K and a slower receptor pathway that bypasses Gi and PI3K. On the other hand, the active platelet factor may initiate a slower signal-transduction event after internalization. The proposed active platelet factor LPA appears to function via receptor activation as it is not active when microinjected into cells (36). Another candidate for the active platelet factor is sphingosine 1-phosphate, a phospholipid, which also mimics the activity of PCM and LPA (J. T. Roberts, P. A. Vincent, C. A. Morton, and F. L. Minnear, unpublished observations). Sphingosine 1-phosphate activates similar G protein-coupled receptors as does LPA and has been reported to be active after internalization (36), although this latter effect is controversial (17).

Because inhibitors of the Gi protein and PI3K significantly modified the permeability-decreasing activity of PCM and are known to modify the activity of ERK, the next logical step was to determine whether ERK was involved. MEK, the upstream kinase of ERK, was inhibited with PD-98059 at 10 and 30 μM or with 10 μM U-0126. These doses of PD-98059 and U-0126 had no effect on PCM activity. However, these doses of PD-98059 and U-0126 lowered the basal phosphorylation of ERK and prevented the PCM-induced increase in ERK phosphorylation. The lack of a correlation between the effects of PD-98059 and U-0126 on electrical resistance and ERK phosphorylation indicates that PCM does not function via ERK to increase endothelial electrical resistance.

Taken together, these results indicate that PCM rapidly increases endothelial electrical resistance via a novel, signal transduction pathway involving tyrosine kinases, the Gi protein, and PI3K. However, PCM can also initiate a signaling cascade downstream or parallel to the Gi protein or P13 kinase to cause a slower increase in endothelial electrical resistance. This novel signaling pathway does not involve PKA, PKG, or ERK.
Substitution of 1-oleoyl-LPA (10 μM) for PCM in the above protocols involving cAMP and inhibitors of the G_i protein and PI3K yielded similar results. The only exception was that pertussis toxin, the inhibitor of G_i, prevented the activity of LPA and delayed the activity of PCM. Interestingly, sphingosine 1-phosphate was also affected by the above experimental protocols in the same way as LPA (unpublished observations).

The cellular mechanism for this endothelial barrier activity of PCM has not been studied. cAMP-enhancing agents also tighten the endothelial barrier and the cellular mechanism is not clear, although some of the cellular targets are known. It has been proposed that cAMP-enhancing agents affect cellular targets that initiate cell spreading and alter the adherens junction proteins, cell-specific cadherins and catenins, that mechanically hold cells together. This is supported by studies showing that an increase in intracellular cAMP causes the inactivation of myosin light-chain kinase (5, 33), a decrease in cellular isometric tension (25), remodeling of actin filaments (16), and cell spreading. A decrease in isometric tension and cell spreading by cAMP could counteract the ability of agents such as thrombin, which increase cellular isometric tension and cause cell contraction, to loosen the endothelial barrier. However, Moy et al. (25) directly measured cellular isometric tension as well as myosin light-chain phosphorylation and concluded that cAMP-enhancing agents affect cellular targets that function to decrease in isometric tension and cell spreading by 33%, a decrease in cellular isometric tension (25), and to increase by 2 h the peripheral localization of epithelial (E)-cadherin (26, 27) and to increase by 2 h the peripheral localization of E-cadherin in brain microvascular endothelial cells (29). Sphingosine 1-phosphate, a lysophospholipid that is synthesized in platelets (40) and that mimics the PCM-induced increase in endothelial electrical resistance (unpublished observations), has been reported to increase within 1 h the localization of vasoendothelial (VE)-cadherin and α-, β-, and γ-catenins to cell-cell contacts of human umbilical vein endothelial cells (17). Microinjection of Clostridium botulinum C3 exoenzyme to inhibit Rho or microinjection of dominant-negative Rac reduced the peripheral localization of VE-cadherin and β-catenin by sphingosine 1-phosphate in these cells. Therefore, PCM as well as cAMP-enhancing agents may function to tighten the endothelial barrier at the level of the actin cytoskeleton and/or the adherens junction.

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