The actin cytoskeleton facilitates complement-mediated activation of cytosolic phospholipase A2

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Cybulsky, Andrey V., Tomoko Takeko, Joan Papillon, Abdelkrim Khadir, Krikor Bijian, and Ludmilla Le Berre. The actin cytoskeleton facilitates complement-mediated activation of cytosolic phospholipase A2. Am J Physiol Renal Physiol 286: F466–F476, 2004. First published November 25, 2003; 10.1152/ajprenal.00260.2003.—Cytosolic PLA2-α (cPLA2) and metabolites of arachidonic acid (AA) are key mediators of complement-dependent glomerular epithelial cell (GEC) injury. Assembly of C5b-9 increases cytosolic Ca2+-concentration and results in transactivation of receptor tyrosine kinases and activation of PLC-γ and the 1,2-diacylglycerol (DAG)-PKC pathway. Ca2+- and PKC are essential for membrane association and increased catalytic activity of cPLA2. This study addresses the role of the actin cytoskeleton in cPLA2 activation. Depolymerization of F-actin by cytochalasin D or latrunculin B reduced complement-dependent [3H]AA release, as well as the complement-induced increase in cPLA2 activity. These effects were due to inhibition of [3H]DAG production and PKC activation, implying interference with PLC. Cytoskeletal inhibitory [3H]AA release was also reduced by jasplakinolide, a compound that stabilizes F-actin and organizes actin filaments at the cell periphery, and calycin A, which induces condensation of actin filaments at the plasma membrane. The latter drugs did not affect [3H]DAG production, suggesting their inhibitory actions were downstream of PKC. Neither cytochalasin D, latrunculin B, nor calycin A affected association of cPLA2 with microsomal membranes, and cytochalasin D and latrunculin B did not alter the localization of the endoplasmic reticulum. Stable transfection of constitutively active RhoA induced formation of stress fibers, stabilized F-actin, and attenuated the complement-induced increase in [3H]AA. Thus in GEC, cPLA2 activation is dependent, in part, on actin remodeling. By regulating complement-mediated activation of cPLA2, the actin cytoskeleton may contribute to the pathophysiology of GEC injury.

inflammation; lipid mediators; protein kinases; signal transduction

ACTIVATION OF THE COMPLEMENT cascade near a cell surface results in the assembly of terminal components, exposure of hydrophobic domains, and insertion of the C5b-9 membrane attack complex into the lipid bilayer of the plasma membrane (20, 22). The consequences of C5b-9 assembly include formation of transmembrane channels or rearrangement of membrane lipids, with the loss of membrane integrity. Nucleated cells require multiple C5b-9 lesions for lysis, whereas, at lower doses, C5b-9 induces sublethal (sublytic) injury and various metabolic effects (20, 22, 31–33). Sublytic C5b-9-mediated cell injury plays a key role in the pathogenesis of passive Heymann nephritis (PHN) in the rat, a widely accepted model of human membranous nephropathy (38). In PHN, antibody seems to be directed against a subset of C5b-9. Antibody treatment significantly reduces proteinuria, but not all antibodies reduce proteinuria in PHN and in human membranous nephropathy (5, 37). Second, cPLA2 may mediate GEC injury more directly, by inducing cell membrane phospholipid hydrolysis and the endoplasmic reticulum (ER) stress response (8).

In a number of cells, cPLA2 is regulated by [Ca2+]i and phosphorylation (11, 13). It has been proposed that an increase in [Ca2+]i into the submicromolar range induces translocation of cPLA2 from the cytosol to an intracellular membrane, where cPLA2 would bind via its NH2-terminal Ca2+-dependent lipid binding or C2 domain, gaining access to phospholipid substate. Phosphorylation on Ser505 increases the catalytic activity of cPLA2. In GEC, cPLA2 is the major endogenous PLA2 isoform. C5b-9 increases free AA, and the release of AA is amplified by overexpression of cPLA2 (23). In GEC, cPLA2 localizes and hydrolyzes phospholipid substrates in the plasma membrane, the membrane of the ER, and the nuclear envelope, but not at mitochondria or the Golgi apparatus (18). Thus the activation of cPLA2 and release of AA are compartmentalized to specific organelles. In GEC, complement enhances cPLA2 phosphorylation and catalytic activity via PLC activation, production of 1,2-diacylglycerol (DAG), and activation of the PKC pathway (23). Mutation of the cPLA2 ERK phosphorylation site (Ser505 to Ala) or pharmacological inhibition of ERK did not reduce cPLA2-mediated AA release, but cPLA2-Ser505-to-Ala mutation nevertheless required the action of PKC (6). Thus in GEC, the role of PKC in complement-dependent cPLA2 activation is essential, but ERK appears to be redundant.

Our earlier studies indicate that the stimulation of cPLA2 activity and AA release is dependent, at least in part, on...
subcellular localization or compartmentalization (18). However, relatively little is known about the organization of signaling cascades activated by C5b-9. Such organization/compartmentalization of phospholipases and protein kinases may be dependent on the cytoskeleton, specifically the actin filament network. The purpose of this study was to examine the role of the actin cytoskeleton in transmission of signals by the C5b-9 complex. We demonstrate that complement-mediated activation of cPLA2 in GEC is regulated by the actin cytoskeleton. The cytoskeleton modulates upstream protein kinase pathways involved in regulating cPLA2 catalytic activity, whereas the membrane association of cPLA2 is unrelated to cytoskeletal integrity.

MATERIALS AND METHODS

Materials. Tissue culture reagents were obtained from Invitrogen Canada (Burlington, ON). Purified C8, complement-deficient sera, cytochalasin D, latrunculin B, calyculin A, PMA, myelin basic protein(4-14) peptide, EGF, and AA were purchased from Sigma (St. Louis, MO). Jasplakinolide was from Molecular Probes (Eugene, OR). [3H]AA (100 Ci/mmol), 1-palmitoyl-2-[arachidonoyl-14C]phosphatidylethanolamine (52 mCi/mmol), and [γ-32P]ATP (3,000 Ci/mmol) were purchased from PerkinElmer Canada (Woodbridge, ON). Mouse anti-phosphotyrosine antibody was from Transduction Laboratories (Lexington, KY). Rabbit anti-cPLA2 and rabbit anti-EGF-R antibodies have been described previously (9, 23). An agaro/se/ conjugated protein consisting of GST and the SH2 and SH3 domains of PLC-γ1 (PLC-γ1-SH2-SH2-SH3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Electrophoresis and immunoblotting reagents were from Bio-Rad Laboratories (Missisauga, ON). Male Sprague-Dawley rats (150 g) were purchased from Charles River Canada (St. Constant, PQ). pRK5-Myc, containing a cDNA encoding a constitutively active mutant of rhodamine A (RhoA; L63/RhoA) (27), was kindly provided by Dr. Natalie Lamarche-Vane (McGill University, Montreal, QC).

Cell culture and transfection. Rat GEC culture and characterization have been published previously (6, 9, 10, 23). GEC were cultured in K1 medium, and studies were done with cells between passages 8 and 60. GEC that stably overexpress cPLA2 were employed in all experiments, except those involving L8/RhoA. Production and characterization of the GEC that stably overexpress cPLA2 were described previously (23). GEC that express L8/RhoA were produced by stable transfection using a method analogous to that for cPLA2.

Incubation of GEC with complement. The standard protocol involved incubation of GEC in monolayer culture with rabbit anti-EGF or sheep anti-Fx1A antisera (5% vol/vol) in modified Krebs-Henseleit buffer (115 mM NaCl, 5 KCl, 0.5 MgSO4, 1 Na2HPO4, 0.5 CaCl2, 5 glucose, and 20 HEPES, pH 7.4, for 40 min at 22°C (6, 9, 10, 23). GEC were then incubated with sublytic normal human serum (NS; diluted in Krebs-Henseleit buffer) or heat-inactivated (at 60°C for 30 min) in control for 40 min at 37°C. In some experiments, antibody-sensitized GEC were incubated with C8-deficient human serum, or C8-deficient serum supplemented with purified C8 (80 μg/ml) diluted serum). As in previous studies, we have generally used heterologous complement to minimize possible signaling via complement-regulatory proteins, although we have demonstrated that homologous complement induces A2 release as well (6, 9, 10, 23). Previous studies have shown that, in GEC, complement is not activated in the absence of antibody (6, 9, 10, 23).

Induction of PHN in rats. PHN was induced by a single intravenous injection of 0.4 ml of sheep anti-Fx1A antisera, as described previously (8, 9). Urine was collected on day 14, and rats were then killed and glomeruli were isolated by differential sieving. All studies were approved by the McGill University Animal Care Committee.

Measurement of free [3H]AA, [3H]DAG, cPLA2 activity, and PKC activity. GEC phospholipids were labeled to isotopic equilibrium with [3H]AA for 48–72 h, as detailed previously (23). Lipids were extracted from ~1 × 106 cells and cell supernatants. Methods of extraction and separation of radiolabeled lipids (e.g., [3H]AA) by thin-layer chromatography are published elsewhere (23). cPLA2 activity was measured using an in vitro assay that monitors release of [3H]AA from [3H]phosphatidylethanolamine (23). PKC activity was determined by measuring phosphorylation of myelin basic protein (MBP, 4-14) peptide, as described previously (6).

Immunoprecipitation and immunoblotting. Preparation of GEC and glomerular lysates and cell fractions was described previously (18, 23). After incubation with antibody and complement, ~6 × 106 GEC were lysed, and proteins were immunoprecipitated with primary antisera, as described previously (6, 9). Immune complexes were incubated with agarose-coupled protein A. For analysis of the EGF-R interaction with PLC-γ1, ~2 × 107 GEC were lysed, and incubated with agarose-conjugated GST-PLC-γ1-SH2-SH2-SH3 fusion protein (4 μg) for 3 h at 4°C. Complexes were boiled in Laemml sample buffer and subjected to SDS-PAGE under reducing conditions. Proteins were then electrophoretically transferred onto nitrocellulose paper, blocked with 3% BSA/2% ovalbumin, and incubated with primary antibody and then with horseradish peroxidase-conjugated secondary antibody. The blots were developed using the enhanced chemiluminescence technique (Amersham Pharmacia Biotech). Protein content was quantified by scanning densitometry, using National Institutes of Health Image software. Preliminary studies demonstrated that there was a linear relationship between densitometric measurements and the amounts of protein loaded onto gels.

Immunofluorescence microscopy. Cells adherent to glass covergrips were fixed with 3% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 (18). After being washed, cells were incubated with rhodamine-phalloidin as described previously (3, 35). Incubation of GEC with membrane-permeant inhibitors of actin polymerization that act by depolymerization of F-actin (Fig. 1). Cytochalasin D and latrunculin B are membrane-permeant inhibitors of actin polymerization that act by depolymerization of F-actin (Fig. 1). Therefore, we...
employed 1 μM latrunculin B and/or 20 μM cytochalasin D concentrations in subsequent experiments. Incubation of antibody-sensitized GEC with a sublytic concentration of NS (to form C5b-9) for 40 min did not alter the staining of rhodamine-phalloidin significantly (not shown). To exclude the possibility that the changes associated with cytochalasin D or latrunculin B were due to cytotoxic effects, GEC were incubated with serially increasing concentrations of complement in the presence or absence of the two drugs. Complement lysis was not affected significantly by the addition of either drug (Table 1).

**Table 1. Effect of cytochalasin D and latrunculin B on complement lysis**

<table>
<thead>
<tr>
<th>NS</th>
<th>LDH Release, % Specific Release</th>
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<tbody>
<tr>
<td></td>
<td>Untreated</td>
</tr>
<tr>
<td>15%</td>
<td>28±2</td>
</tr>
<tr>
<td>10%</td>
<td>22±1</td>
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<td>5%</td>
<td>12±2</td>
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Values are means ± SE. LDH, lactate dehydrogenase; NS, normal human serum. Glomerular epithelial cells (GEC) were untreated, or pretreated with cytochalasin D (20 μM) or latrunculin B (1 μM) for 30 min at 37°C. Then, GEC were incubated with antibody, followed by 5, 10, or 15% NS (heat-inactivated human serum (HIS) in control). LDH release (reflecting cell lysis) was determined after 40 min. There are no statistically significant differences among the untreated or treated groups (3 experiments).
GST fusion protein that contains the SH2 and SH3 domains of PLC-γ1. GST-PLC-γ1-SH2-SH2-SH3 bound to EGF-R only in lysates of complement-treated GEC, and the bound EGF-R was tyrosine phosphorylated (Fig. 2B). Previously, we also showed that complement induces tyrosine phosphorylation of PLC-γ1 (6). A result analogous to PLC-γ1 was obtained using a GST-Grb-2 fusion protein in an earlier study (9).

The above experiments demonstrate that complement induces EGF-R transactivation in cultured GEC, but it is important to determine whether analogous changes occur in C5b-9-mediated GEC injury in vivo. To address this question, we assessed EGF-R tyrosine phosphorylation and protein expression in the PHN model of membranous nephropathy, where GEC injury and proteinuria are due to C5b-9 assembly. Glomeruli were isolated from control rats and from rats with PHN on day 14, a time point when these rats show marked proteinuria (8). Glomerular EGF-R phosphorylation was enhanced about twofold in rats with PHN, compared with control, and there were no differences in EGF-R protein expression (Fig. 3).

In earlier studies, we demonstrated that signals downstream of EGF-R, including cPLA2, are also activated in PHN (10). Together, the results imply that cultured GEC reflect pathophysiological changes in vivo.

An intact actin cytoskeleton is required for complement-induced stimulation of cPLA2 catalytic activity and release of AA. In keeping with previous results, incubation of antibody-sensitized GEC with complement (NS) induced an increase in free [3H]AA (Fig. 4A). Furthermore, incubation with C8-deficient serum reconstituted with purified C8 increased free [3H]AA (5.47 ± 0.47% of total radioactivity) compared with unreconstituted C8-deficient serum (1.57 ± 0.42% of total radioactivity), indicating that release of AA is due to assembly of C5b-9 (8). The complement-induced release of AA is dependent on the activation of cPLA2 (23), and the mechanism involves Ca2+-dependent association of cPLA2 with membranes of GEC organelles (18, 23), as well as an increase in cPLA2 catalytic activity due to phosphorylation via a PKC-dependent pathway (6, 18, 23). Complement-induced [3H]AA release was inhibited by ~45% with cytochalasin D and by ~80% with latrunculin B (Fig. 4, A and B). Disruption of the cytoskeleton may have reduced [3H]AA release either by interfering with the association of cPLA2 with membranes or by inhibiting the catalytic activity of cPLA2. To distinguish between these possibilities, we first employed an in vitro cPLA2 activity assay that monitors release of [14C]AA from

Fig. 2. Complement induces EGF-R receptor (EGF-R) activation (representative immunoblots). A: EGF-R tyrosine phosphorylation and protein expression. GEC were untreated or pretreated with cytochalasin D (CD; 20 μM) or latrunculin B (LB; 1 μM) for 30 min at 37°C; GEC were incubated with antibody and 2.5% normal human serum (NS) or with heat-inactivated human serum (HIS) in control. Alternatively, GEC were incubated with 2.5% C8-deficient serum reconstituted with purified C8 (C8DS+c8) or C8DS alone. Lysates were immunoprecipitated with anti-EGF-R antiserum, and the immune complexes were blotted with anti-phosphotyrosine (PY) or anti-EGF-R antibodies. The tyrosine-phosphorylated EGF-R bound to PLC-γ1-SH2-SH2-SH3, and the complexes were blotted with anti-PY or anti-EGF-R antibodies. The tyrosine-phosphorylated EGF-R bound to PLC-γ1-SH2-SH2-SH3 in GEC treated with complement or EGF.

Fig. 3. EGF-R tyrosine phosphorylation is enhanced in glomeruli of rats with passive Heymann nephritis (PHN). Glomeruli were isolated from normal (control) rats and proteinuric rats with PHN on day 14. Lysates were immunoprecipitated with anti-EGF-R antiserum, and the immune complexes were blotted with anti-PY or anti-EGF-R antibodies. The tyrosine-phosphorylated EGF-R bound to PLC-γ1-SH2-SH2-SH3 in GEC treated with complement or EGF.
Complement-induced activation of cPLA₂ is inhibited by drugs that alter the cytoskeleton. GEC that overexpress cPLA₂ were labeled with [³H]arachidonic acid (AA; A, B, D–F). GEC were untreated or pretreated with CD (20 μM; A and C), LB (1 μM; B and E), jasplakinolide (JP; 10 μM; D and E), or calyculin A (CA; 20 or 100 nM; F) for 30 min at 37°C. To deplete PKC (A), GEC were preincubated with PMA (2 μM) for 18–24 h. GEC were incubated with antibody and 2.5% NS (to form sublytic CsB-9) or HIS in controls. Lipids were extracted (A, B, D–F) and analyzed by thin-layer chromatography. C: cytosolic fractions were prepared, and PLA₂ activity was measured by release of [³H]AA from exogenous 1-palmitoyl-2-[¹⁴C]arachidonoyl phosphatidylethanolamine. CD (A), LB (B), JP (D), and CA (100 nM; F) significantly inhibited the complement-induced increase in free [³H]AA. Release of [³H]AA was also blocked in PKC-depleted GEC (A). CD inhibited complement-stimulated cPLA₂ catalytic activity (C). Basal PLA₂ activity (i.e., HIS group) was 331 ± 76 pmol/min·mg protein⁻¹ (C). Basal [³H]AA levels ranged from 0.9 to 1.7% of total radioactivity (D–F). *P < 0.0001 NS vs. HIS, P < 0.015 NS vs. NS+CD, P < 0.015 NS vs. NS-PKC depleted (4–9 experiments performed in duplicate). **P < 0.01 NS vs. HIS, P < 0.03 NS vs. NS+LB (4 experiments performed in duplicate). ***P < 0.001 NS vs. HIS, P < 0.01 NS vs. NS+CD (3 experiments performed in duplicate). *P < 0.0001 NS vs. HIS, P < 0.001 NS vs. NS+JP (8 experiments performed in duplicate). **P < 0.002 NS vs. HIS (3 experiments performed in duplicate). *P < 0.002 NS vs. HIS, P < 0.04 NS vs. HS+CA-100 (5–6 experiments performed in duplicate).

In keeping with prior results, it was demonstrated that cPLA₂ activity was stably increased in lysates of GEC that had been incubated with antibody and complement, compared with control (Fig. 4C). Pretreatment of GEC with cytochalasin D reduced basal PLA₂ activity and abolished the complement-stimulated increase. The inhibitory effect of cytochalasin D on PLA₂ activity appeared to be greater than its effect on AA release in intact cells, but it should be noted that the two assays measure complementary, although not identical, parameters (e.g., the in vitro assay does not reflect the role of Ca²⁺ in AA release). The cPLA₂ activity assay was also employed to address the effect of latrunculin B (protocol as in Fig. 4C). In the absence of latrunculin B pretreatment, complement stimulated an increase in cPLA₂ activity in GEC extracts that was 2.58 ± 0.47-fold of control (P < 0.015). After pretreatment with 1 μM latrunculin B, the complement-stimulated increase in cPLA₂ activity was trivial (0.07 ± 0.15-fold of control; 3 experiments). Thus the effect of latrunculin B was in keeping with that of cytochalasin D.

Unlike cytochalasin D or latrunculin B, which depolymerize the actin cytoskeleton, jasplakinolide is a cell-permeant compound that stabilizes F-actin and organizes actin filaments at the cell periphery, near the plasma membrane (29). (Because jasplakinolide competes with phalloidin for the F-actin binding site, it is not possible to examine rhodamine-phalloidin staining in cells after jasplakinolide treatment.) We predicted that preincubation of GEC with jasplakinolide may enhance the complement-induced increase in free [³H]AA, but contrary to expectations, jasplakinolide inhibited the release of free [³H]AA by complement (Fig. 4D) and did not reverse the inhibitory effect of latrunculin B (Fig. 4E). Phosphorylation of the ezrin-radixin-moesin family of proteins is required for cross-linking of actin to the plasma membrane (41). GEC were treated with calyculin A to induce phosphorylation-dependent association of these proteins with the plasma membrane. Calyculin A is a serine/threonine protein phosphatase inhibitor that inhibits phosphatases 1 and 2, and treatment of many cell lines with calyculin A results in a condensation of actin filaments at the plasma membrane (1, 29). Preincubation of GEC with calyculin A inhibited the complement-induced increase in free [³H]AA (Fig. 4F). Calyculin A also partially inhibited the complement-induced stimulation of cPLA₂ activity, measured by release of [¹⁴C]AA from exogenously added phospholipid substrate in vitro (complement: 152 ± 15% of...
Fig. 5. Effect of cytoskeletal disruption on \(^{3}\text{H}\)-diacylglycerol (DAG) production and PKC activation. GEC were labeled with \(^{3}\text{H}\)AA (A). GEC were untreated or pretreated with CD (20 \(\mu\)M), LB (1 \(\mu\)M), IP (10 \(\mu\)M), or CA (100 nM) and then with antibody and complement, as in Fig. 4. A: lipids were extracted and analyzed by thin-layer chromatography. Complement increased \(^{3}\text{H}\)DAG significantly. The stimulatory effect of complement was blocked by LB and, in part, by CD, but not by JP or CA.* \(P < 0.001, \) ** \(P < 0.015\) vs. HIS (9 experiments performed in duplicate). **P \( < 0.02\) vs. HIS (4 experiments performed in duplicate). ***P \( < 0.01\), \( \times P < 0.02\) vs. HIS (4 experiments performed in duplicate). B: PKC activity was measured in situ by monitoring phosphorylation of myelin basic protein (4-14) peptide. Complement stimulated PKC activity significantly. LB blocked the stimulatory effect of complement by 100%, whereas CD produced a 35% inhibition. The stimulatory effect of PMA (250 ng/ml) is shown for comparison. \( * P < 0.015\) vs. HIS (6 experiments).

control, complement + calyculin A: 130 \(\pm\) 20% of control, \(P < 0.005\), 5 experiments).

Disruption of the actin cytoskeleton affects pathways upstream of cPLA2. The next series of experiments assessed whether the actions of the compounds that affect the cytoskeleton were directed specifically at cPLA2 or at upstream mediators, i.e., production of \(^{3}\text{H}\)DAG and/or activation of PKC. In GEC, complement increases inositol trisphosphate and DAG (\(^{3}\text{H}\)DAG and DAG mass) (4, 7), and the complement-induced activation of cPLA2 is dependent on the activation of the DAG-PKC pathway, but is independent of ERK (6). In keeping with previous results, incubation of GEC with complement increased \(^{3}\text{H}\)DAG and PKC activity (Fig. 5, A and B), and depletion of PKC reduced the complement-mediated increase in free \(^{3}\text{H}\)AA by \( \sim\)75% (Fig. 4A). Latrunculin B blocked both the complement-induced increases in \(^{3}\text{H}\)DAG and PKC activity almost completely (Fig. 5, A and B). Cytochalasin D reduced the complement-induced increases in \(^{3}\text{H}\)DAG and PKC activity by \( \sim\)35%, although the increase in \(^{3}\text{H}\)DAG remained statistically significant (Fig. 5, A and B). Thus changes in \(^{3}\text{H}\)DAG correlate closely with changes in PKC activity, and depolymerization of the actin cytoskeleton reduces \(^{3}\text{H}\)DAG production (and PKC activation) by blocking PLC-mediated phospholipid hydrolysis. Although jasplakinolide and calyculin A blocked the complement-dependent increase in free \(^{3}\text{H}\)AA, these compounds had no effect on changes in \(^{3}\text{H}\)DAG (Fig. 5A) or PKC activity (not shown).

To further delineate the sites of action of cytoskeleton-altering drugs, we studied the effects of PMA on the release of \(^{3}\text{H}\)AA. In these experiments, we employed an experimental model developed earlier, in which GEC are first incubated with PMA (to activate PKC), and then \([\text{Ca}^{2+}]_i\) is clamped by permeabilizing GEC with buffers containing specific concentrations of \([\text{Ca}^{2+}]_i\), as PMA does not independently increase \([\text{Ca}^{2+}]_i\), in GEC (6). Permeabilization of untreated GEC with buffer containing 1 mM free \(\text{Ca}^{2+}\) induced an upward trend in free \(^{3}\text{H}\)AA, compared with 0.1 \(\mu\)M free \(\text{Ca}^{2+}\) (resting \([\text{Ca}^{2+}]_i\); Fig. 6). A greater increase in free \(^{3}\text{H}\)AA was induced by treatment of GEC with PMA, plus permeabilization with buffer containing 1 mM \(\text{Ca}^{2+}\) (Fig. 6). Pretreatment of GEC with latrunculin B had no significant inhibitory effect on the PMA-induced increase in free \(^{3}\text{H}\)AA (Fig. 6). Similarly, cytochalasin D had no effect on \(^{3}\text{H}\)AA release by PMA (4-7 experiments; data not shown). In contrast to cytochalasin D and latrunculin B, pretreatment of GEC with jasplakinolide or calyculin A inhibited the \([\text{Ca}^{2+}]_i\) - and PMA-stimulated release of \(^{3}\text{H}\)AA by \( \sim\)100% (5-6 experiments; data not shown). Therefore, the inhibitory effects of latrunculin B and cytochalasin D on complement-induced activation of cPLA2 are at least, in part, to inhibition of steps upstream of PKC, involving inhibition of PLC (Fig. 5), whereas jasplakinolide and calyculin A most likely inhibit the action of PKC or PKC effectors on cPLA2 activity.

Disruption of the actin cytoskeleton does not affect the membrane association of cPLA2. The above experiments indicate that disruption of the cytoskeleton leads to inhibition of complement-stimulated cPLA2 catalytic activity. In the next series of experiments, we examined whether cytoskeleton-disrupting drugs affected the association of cPLA2 with membranes, which is an essential step for the release of AA. In an earlier study, we demonstrated that in resting GEC, a portion of cPLA2 was associated with the membrane (microsomal) fraction and that, in complement-stimulated GEC, the majority of phospholipid hydrolysis occurred at the ER (18). However, translocation of cPLA2 from the cytosol to the membrane compartment was not detected with a physiological agonist, such as CSb-9, but only after incubation of GEC with the \(\text{Ca}^{2+}\) ionophore ionomycin (which induces a greater increase in outer monolayer and cytosol). In these experiments, cPLA2 activity was assessed in the presence of [\(\text{Ca}^{2+}\)]_o (4, 7), and the complement-induced increase in free \(^{3}\text{H}\)AA, compared with 0.1 \(\mu\)M free \(\text{Ca}^{2+}\) (resting \([\text{Ca}^{2+}]_i\); Fig. 6). A greater increase in free \(^{3}\text{H}\)AA was induced by treatment of GEC with PMA, plus permeabilization with buffer containing 1 mM \(\text{Ca}^{2+}\) (Fig. 6). Pretreatment of GEC with latrunculin B had no significant inhibitory effect on the PMA-induced increase in free \(^{3}\text{H}\)AA (Fig. 6). Similarly, cytochalasin D had no effect on \(^{3}\text{H}\)AA release by PMA (4-7 experiments; data not shown). In contrast to cytochalasin D and latrunculin B, pretreatment of GEC with jasplakinolide or calyculin A inhibited the \([\text{Ca}^{2+}]_i\) - and PMA-stimulated release of \(^{3}\text{H}\)AA by \( \sim\)100% (5-6 experiments; data not shown). Therefore, the inhibitory effects of latrunculin B and cytochalasin D on complement-induced activation of cPLA2 are at least, in part, to inhibition of steps upstream of PKC, involving inhibition of PLC (Fig. 5), whereas jasplakinolide and calyculin A most likely inhibit the action of PKC or PKC effectors on cPLA2 activity.
In untreated GEC, cPLA₂ was found in both cytosolic and microsomal fractions, whereas the ER protein calnexin (18) was exclusively microsomal (Fig. 7A). Cytochalasin D and latrunculin B did not affect the amount of cPLA₂ recovered in the microsomal fraction (Fig. 7A). In a second set of experiments, GEC were untreated, or treated with ionomycin (to facilitate cPLA₂ translocation) or ionomycin plus calyculin A. Ionomycin increased the amount of cPLA₂ in the microsomal fraction, and this increase was not affected by CA. C: cPLA₂ does not associate with the cytoskeleton. GEC were untreated or treated with ionomycin (10 μM)+PMA (250 ng/ml). Cells were incubated in buffer containing 1% Triton X-100 for 10 min. The cytoskeleton was then solubilized with buffer containing 1% Triton X-100+1% deoxycholate. cPLA₂ is found almost exclusively in the Triton-soluble fractions.

[Ca²⁺], compared with C5b-9. In untreated GEC, cPLA₂ was found in both cytosolic and microsomal fractions, whereas the ER protein calnexin (18) was exclusively microsomal (Fig. 7A). Cytochalasin D and latrunculin B did not affect the amount of cPLA₂ recovered in the microsomal fraction (Fig. 7A). In a second set of experiments, GEC were untreated, or treated with ionomycin (to facilitate cPLA₂ translocation) or ionomycin plus calyculin A. Ionomycin induced a small increase in microsomal cPLA₂, compared with untreated cells, but by analogy to cytochalasin D and latrunculin B, calyculin A did not affect the microsomal association of cPLA₂ (Fig. 7B).

The effects of latrunculin B and cytochalasin D on cPLA₂ were also studied using immunofluorescence microscopy (Fig. 8A). In GEC, cPLA₂ staining was predominantly cytosolic, and in some cells (particularly after treatment with the Ca²⁺ ionophore A-23187), there was perinuclear enhancement, in keeping with localization at the membrane of the ER or nuclear envelope (18). The perinuclear enhancement was not affected by pretreatment with latrunculin B (Fig. 8A). Similarly, cytochalasin D did not affect cPLA₂ staining (results not shown). GEC were also stained with antibody to calnexin, to localize the ER (Fig. 8B). The distribution of calnexin in resting cells was mainly perinuclear, with some extension of calnexin from the nucleus toward the cell periphery. Calnexin staining was not affected by A-23187. Treatment with latrunculin B (Fig. 8B) or cytochalasin D (not shown) did not have any significant effect on the perinuclear staining pattern of calnexin, but there was slightly less peripheral extension of the staining from the perinuclear regions. These results indicate that latrunculin B or cytochalasin D, while altering the actin cytoskeleton (Fig. 1), did not alter the perinuclear localization of the ER and cPLA₂ (Fig. 8), and, together with the biochemical data (Fig. 7, A and B), suggests that ER-cPLA₂ interaction remains intact despite cytoskeletal disruption.
Finally, we assessed whether cPLA₂ was associated with the actin cytoskeleton. In these experiments, GEC were incubated with or without ionomycin + PMA and were then treated with buffer containing 1% Triton X-100. Almost all of the cPLA₂ was recovered in the Triton-soluble fraction (Fig. 7C), and only trivial amounts were present in the Triton-insoluble (cytoskeleton) fraction, suggesting no significant association of cPLA₂ with the cytoskeleton.

Effect of L showdown RhoA expression on the cytoskeleton and AA release. Rho GTPases are known to stabilize actin filaments and induce stress fiber formation in various cells (2). In GEC, stable expression of a constitutively active RhoA mutant (L showdown RhoA) (Fig. 9A) resulted in increased actin polymerization, as reflected by the appearance of stress fibers superimposed on the cortical distribution of F-actin (Fig. 1E). In addition, while stress fibers disappeared after incubation with latrunculin B, the L showdown RhoA-transfected GEC showed a relative resistance to depolymerization of cortical actin by latrunculin B, compared with Neo GEC (Fig. 1F). Complement-induced increases in [³H]AA and [³H]DAG were attenuated significantly in the L showdown RhoA-transfected cells compared with Neo GEC (Fig. 9B). The association of cPLA₂ with the microsomal membrane fraction did not appear to be significantly different between the GEC that overexpress L showdown RhoA and Neo GEC (Fig. 9C), suggesting that L showdown RhoA acted via attenuation of the complement-mediated stimulation of cPLA₂ catalytic activity. We were not able to verify directly that L showdown RhoA attenuated cPLA₂ activity, because the GEC that overexpress L showdown RhoA express endogenous cPLA₂ (i.e., these cells do not overexpress cPLA₂), and changes in endogenous cPLA₂ activity were too small to be quantitated reliably in the in vitro PLA₂ assay.

DISCUSSION

In this study, we have demonstrated that complement-induced activation of cPLA₂ is dependent on the actin cytoskeleton. Transmission of signals by C5b-9 is initiated at the plasma membrane, at least in part, via transactivation of EGF-R (9) (Fig. 2). This mechanism operates in both cultured GEC and the PHN model of C5b-9-dependent GEC injury in vivo (Fig. 3). EGF-R transactivation occurred independently of cytochalasin D or latrunculin B (Fig. 2) and resulted in activation of PLC-γ1 (Fig. 3), production of DAG, and activation of PKC (Fig. 5). Both PKC activation and a C5b-9-induced increase in [Ca²⁺]ᵢ are essential for activation of cPLA₂ in GEC (6, 23). The complement-induced increase in cPLA₂ activity and [³H]AA release was inhibited by cytochalasin D and latrunculin B (Fig. 4). The two drugs also inhibited complement-induced increases in [³H]DAG and PKC activity (Fig. 5), indicating that cytochalasin D and latrunculin B most likely inhibited cPLA₂ activity indirectly, at least in part, by blocking the activation or function of PLC-γ1. The actions of cytochalasin D and latrunculin B on [³H]AA release were similar, but latrunculin B generally exhibited a greater inhibitory effect. Actually, it has been reported that latrunculins may be more potent agents than cytochalasin D (35). In addition, cytochalasin D and latrunculin induce actin cytoskeleton depolymerization through different mechanisms. Cytochalasin D binds to the barbed (growing end) of actin filaments and prevents actin filament formation or leads to disruption of actively turning over actin stress fibers. Latrunculins sequester G-actin monomers preventing actin polymerization and effectively disrupt both actin stress fibers, as well as cortical actin filaments, which are more resistant to cytochalasin D (19). In GEC, the actin filaments tended to be distributed in a cortical pattern (Fig. 1), consistent with the more potent effect of latrunculin B. In other experiments (unpublished observations), we demonstrated that complement-induced activation of ERK.
was inhibited by disruption of the cytoskeleton, but activation of c-Jun NH2-terminal kinase was not. Thus pharmacological disassembly of the actin filament network does not exert a general inhibitory effect on all signaling pathways.

Stabilization of actin polymerization by jasplakinolide reduced the complement-induced increase in \[^{3}H\]AA release (Fig. 4). The same effect was observed with calyculin A, which condenses actin filaments at the cell periphery near the plasma membrane. Moreover, calyculin A partially inhibited the complement-induced increase in cPLA2 activity (we were not able to directly test the effect of jasplakinolide on cPLA2 activity for practical reasons). Jasplakinolide and calyculin A blocked the PMA-induced release of \[^{3}H\]AA (in the presence of increased \([Ca^{2+}])\) and did not affect changes in \[^{3}H\]DAG. Therefore, these two drugs most likely interfered with the stimulation of cPLA2 catalytic activity downstream of PKC. Stable expression of L63 RhoA attenuated complement-induced increases in \[^{3}H\]AA and \[^{3}H\]DAG (Fig. 9). This effect was associated with enhanced actin polymerization (Fig. 1E). In addition, L63 RhoA may have acted via a mechanism analogous to calyculin A, because constitutively active RhoA was reported to induce phosphorylation of ezrin-radixin-moesin proteins (2). Together, the results demonstrate that both depolymerization and stabilization of the actin cytoskeleton can reduce AA release and suggest that AA release is dependent on cytoskeletal remodeling (see below).

Our results indicate that the cytoskeleton-disrupting drugs most likely affected signaling events in the vicinity of the plasma membrane. Cytochalasin D and latrunculin B interfered with PLC-γ1, which is believed to function mainly at the plasma membrane, where substrate, i.e., phosphatidylinositol 4,5-bisphosphate (PIP2) is most abundant (44). Integrin engagement by extracellular matrix results in accumulation of talin, focal adhesion kinase, vinculin, and α-actinin around the integrin cytoplasmic domain. Actin filaments interact with α-actinin and talin to form a supporting structure that organizes focal contacts. F-actin assembly may be dependent on Rho GTPases, Rho kinases, phosphatidylinositol 4-phosphate 5-kinase, as well as ezrin-radixin-moesin proteins (2). An equilibrium exists between PIP2, F-actin, actin-binding proteins (profilin, gelsolin, cofillin), and Rho GTPases, such that changes in actin polymerization could be associated with changes in PIP2 (15, 26). Alternatively, it has been reported that activation of PLC-γ1 by EGF may depend on association with the actin cytoskeleton (46). Production of DAG after PIP2 hydrolysis leads to the activation of PKC. On stimulation of cells, various isoforms of PKC translocate to the plasma membrane (as well as other subcellular sites) (12, 17). The regulation of actin polymerization and its potential role in PKC activation (e.g., Ca\(^{2+}\) and lipid dependence, anchoring proteins) (28) will require further study. Additional studies will also be required to define the relevant PKC isoforms in GEC (14). It should also be noted that in GEC there was no direct association of cPLA2 with the actin cytoskeleton, although cPLA2 has been reported to interact with the intermediate filament protein vimentin (21).

Our previous studies showed that association of cPLA2 with subcellular membranes occurs in resting and stimulated GEC and is essential for AA release (18). The ER is the principal site of phospholipid hydrolysis by cPLA2 and the most important source of free AA. Treatment of GEC with cytochalasin D, latrunculin B, or calyculin A did not alter the amount of cPLA2 associated with microsomal membranes (which include ER), suggesting that an intact actin cytoskeleton is not essential for the association of cPLA2 with the membrane compartment (Fig. 7). Furthermore, the ER (as visualized by calnexin staining) appeared to be unaffected (or affected to only a minor extent) by cytochalasin D and latrunculin B (Fig. 8). Similarly, treatment of hepatocytes with cytochalasin D induced only minor changes in ER ultrastructure (43).

Our study, which has revealed an important role for the actin cytoskeleton in complement signaling, is in keeping with studies in other systems, which showed that the actin cytoskeleton is important in cell cycle progression, including expression of immediate early genes and cyclins (40). In response to insulin treatment, actin filament disassembly blocked activation of Ras, ERK, and p38 mitogen-activated protein kinase, but not insulin receptor autophosphorylation, phosphatidylinositol 3-kinase, or S6 kinase (16, 40). Moreover, binding of Shc to the insulin receptor was not affected, but binding of Grb2 to Shc was disrupted (40). The authors were not able to determine whether there was direct association of Shc or Grb2 with the cytoskeleton. Serum response factor regulates transcription of many serum-inducible genes and is activated by LIM kinase-1. Activation is blocked by latrunculin B (34). In rat mesangial cells, disruption of the actin cytoskeleton with latrunculin B upregulated interleukin-1β-induced expression of inducible nitric oxide synthase, whereas jasplakinolide suppressed the enhancement by latrunculin B. Also, latrunculin B decreased serum response factor activity, and serum response factor played a negative regulatory role in the expression of inducible nitric oxide synthase (47). It would seem that jasplakinolide (which facilitates actin polymerization) should produce an effect opposite to that of cytochalasin D or latrunculin B (which depolymerize the cytoskeleton). However, all of these drugs were inhibitory to AA release in the present study, and parallel effects of jasplakinolide and latrunculin B or cytochalasin D have been reported in several other systems. For example, jasplakinolide and latrunculin B both inhibited insulin-stimulated glucose uptake in adipocytes (16), lipopolysaccharide-mediated production of reactive oxygen species in monocytes (30), and accumulation of phosphatidylinositol 3,4,5-trisphosphate in response to a chemotactic stimulus in neutrophils (42), whereas jasplakinolide and cytochalasin D both induced apoptosis in airway epithelial cells (45).

The protocol employed in the present study did not result in complement-induced changes in F-actin, although we observed that F-actin decreases with more prolonged complement exposure (37). In another study (39), sublethal GEC injury by complement was associated with loss of actin stress fibers and focal contacts, but not integrins. There was a reduction in tyrosine phosphorylation of paxillin but no change in content of focal contact proteins. The complement-induced disassembly of the actin cytoskeleton may have been due to ATP depletion, or loss of other cytosolic components, and recovery from injury was seen in 18 h (39). In vivo, GEC (podocytes) contain F-actin as a thin layer at the base of the foot processes, and abnormalities in actin-associated proteins may lead to a disruption in GEC architecture (24). Podocyte foot process effacement and focal detachment from the glomerular basement membrane are prominent in C5b-9-mediated glomerular injury. This raises the possibility of disassembly of focal adhesion complexes or actin filaments, which support foot
processes. Further studies will be required to determine how the cytoskeleton modulates the pathophysiology of complement-dependent GEC injury in vivo.

REFERENCES


