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Cytosolic calcium and protein kinase C reduce complement-mediated glomerular epithelial injury

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Cytosolic calcium and protein kinase C reduce complement-mediated glomerular epithelial injury. In rat membranous nephropathy, proteinuria is due to formation of the C5b-9 membrane attack complex of complement (C), and is associated with morphological evidence of glomerular epithelial cell (GEC) injury. Analogous morphological changes are induced by C5b-9 in cultured GEC. In addition, in cultured GEC C5b-9 induces Ca^{2+} influx, as well as Ca^{2+} mobilization and increased 1,2-diacylglycerol due to the activation of phospholipase C. In this study we investigated how this GEC activation pattern might influence C-mediated GEC injury. We demonstrate that the C5b-9-induced increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) did not impair ATP generation by mitochondria, suggesting that it does not contribute to cytotoxicity. Moreover, this increase in $[Ca^{2+}]_i$ protected GEC from C-mediated cytolysis. However, a large increase in $[Ca^{2+}]_i$ (produced by the Ca^{2+} ionophore A23187) impaired ATP generation and aggravated C-mediated cytotoxicity, suggesting that intact mitochondrial activity is necessary for GEC to withstand C attack. Activation of protein kinase C (PKC) by phorbol myristate acetate (PMA) also decreased C-mediated cytolysis. Conversely, C lysis was enhanced in GEC that had been pretreated for 18 hours with a high dose of PMA to deplete PKC, and following PKC inhibition with H-7. Therefore, PKC activation, possibly resulting from C5b-9-induced increase in 1,2-diacylglycerol, triggered mechanisms that protected GEC from C-mediated injury. Thus, as a consequence of C5b-9-induced phospholipase activation, the amount of C-induced GEC injury is diminished.

Glomerular capillary wall injury in the passive Heymann nephritis model of rat membranous nephropathy is mediated by antibody (anti-Fx1A) and the C5b-9 membrane attack complex (MAC) of complement (C) [1–3]. The primary target of antibody and C5b-9 is the visceral glomerular epithelial cell (GEC) [2, 4], which suffers noncytolytic injury, appearing as effacement of foot processes and villous transformation and vesiculation of the plasma membranes [2, 4]. Rat GEC in culture are also susceptible to sublytic injury by C5b-9 [5, 6]. On exposure to concentrations of C that fail to release cellular lactate dehydrogenase, GEC, sensitized with anti-Fx1A, release low molecular weight markers [5], and develop membrane vesicles [5] that contain the MAC [6]. These ultrastructural findings in cultured GEC resemble those in the perfused kidney model of Heymann nephritis [2], and are compatible with the recently reported extrusion of MAC-containing vesicles from GEC into the urine of rats with passive Heymann nephritis [4].

Several characteristics of C attack on nucleated cells have been defined in the last decade. In contrast to erythrocytes, nucleated cells are relatively resistant to C lysis, as mechanisms exist that allow nucleated cells to resist or recover from C attack [7–13]. For example, terminal C complexes may be eliminated from the plasma membranes of nucleated cells by endocytosis or exocytosis [7, 8], and this elimination is stimulated by an increase in the cytosolic-free Ca^{2+} concentration ($[Ca^{2+}]_i$) [9–11]. Exocytosis of C5b-9 complexes manifests morphologically as membrane vesiculation [8]. In contrast, inhibition of either protein synthesis [12] or assembly of free fatty acids into cellular lipids [13], and factors that increase osmotic fragility of cells [12], render nucleated cells more susceptible to C lysis. At sublytic concentrations, C5b-9 can activate various cellular processes, including production of reactive oxygen species [12, 14], hydrolysis of phospholipids, release of arachidonic acid and eicosanoids [15–18], and release of interleukin-1 [17]. In addition to nucleated cells, similar actions of C5b-9 have been demonstrated in platelets, some of which include Ca^{2+}-dependent exocytosis of C5b-9 complexes (manifesting as membrane vesiculation) [19], and activation of protein kinase C (PKC) [20].

Our observations in passive Heymann nephritis and in cultured GEC following C attack suggested that assembly of the MAC in rat membranous nephropathy might lead to altered GEC function and proteinuria through activation of cellular processes. Recently we observed that insertion of the MAC into the GEC plasma membranes led to a rapid increase in $[Ca^{2+}]_i$ that was due to both Ca^{2+} influx and Ca^{2+} release from intracellular stores [21]. C5b-9 also stimulated production of inositol trisphosphate, 1,2-diacylglycerol (DAG), free arachidonic acid, prostaglandin $F_{2\alpha}$ and thromboxane A_2 [21]. Thus, in GEC, the MAC activated a phosphatidylinositol-directed phospholipase C and possibly phospholipase A_2. Since Ca^{2+}, DAG and free fatty acids are capable of activating PKC [22], it is reasonable to expect that PKC activity might have also increased following formation of C5b-9. Furthermore, we found that the MAC-induced increases in DAG and arachidonic acid

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could be partly inhibited with indomethacin, suggesting that the MAC-induced release and subsequent metabolites of arachidonic acid amplify the effect of the MAC by further increasing arachidonic acid and DAG [21].

Presently, it is unknown how the cellular activation pattern induced by the MAC leads to altered GEC morphology and to proteinuria in rat membranous nephropathy. Elevated $[Ca^{2+}]_i$ and phospholipase-induced phospholipid breakdown could alter membrane structure and function, such as, by inducing alterations in membrane enzyme activity and loss of ionic gradients [23, 24]. Ca^{2+} uptake into mitochondria can result in uncoupling of oxidative phosphorylation [23]. An increase in $[Ca^{2+}]_i$ could also result in the nucleation of actin filament assembly [24, 25], leading to a breakdown of the cytoskeleton, which in turn could induce changes in plasma membrane structure, including vesiculation. Alternatively, increased $[Ca^{2+}]_i$ and phospholipase activation could stimulate endocytosis or exocytosis of C5b-9 complexes [26, 27], which could serve as a mechanism to protect GEC from C membrane attack [7, 8], and could be an alternate explanation for the vesiculation of the GEC plasma membranes observed in rat membranous nephropathy. In the present study we examined how MAC-induced activation of GEC contributes to C-mediated GEC injury. We observed that phospholipase activation and elevation of $[Ca^{2+}]_i$ trigger mechanisms that protect GEC from C lysis.

Methods

GEC culture

Primary cultures of rat GEC were established as described previously [5, 28]. GEC were grown in tissue culture dishes (Falcon, Beckton Dickinson and Co., Oxnard, California, USA) that were coated with collagen (Vitrogen, Collagen Corp., Palo Alto, California, USA) in Dulbecco's Modified Eagle Medium/Ham F-10 (1:1) (Gibco Laboratories, Grand Island, New York, USA, and Burlington, Ontario, Canada), containing 5% Nu-Serum (Collaborative Research, Bedford, Massachusetts, USA) and hormone supplements [5]. GEC were characterized according to established criteria which include: polygonal shape and cobblestone appearance at confluency, cytotoxic susceptibility to low doses of aminonucleoside of puromycin, positive immunofluorescence staining for cytokeratin, and presence of junctional complexes by electron microscopy [5].

Most studies were performed with cells adherent to a collagen substratum in monolayer culture, except binding of ^{125}I-C9 (see below), which was carried out in GEC in suspension [5]. Cells were used between passages 20 and 60. Despite repeated passage, GEC retained their morphological characteristics and differentiated features of epithelium (including junctional complexes), retained the specificity for binding anti-Fx1A (see below), and demonstrated C-mediated cytotoxicity for >60 passages.

Antibody

The nephritogenic antibody of PHN, anti-rat Fx1A, was used to activate C on the GEC membrane. A sheep was immunized with proximal tubule fraction (Fx1A), as previously described [1, 2]. Antiserum was collected and tested for binding to brush border in kidney sections by indirect immunofluorescence, and for ability to form subepithelial immune deposits and induce proteinuria in rats, as described previously [1]. The C-fixing IgG1 fraction of sheep anti-Fx1A was prepared by ion-exchange chromatography [1].

C components and sensitization of cells

To study effects of Ca^{2+} in C membrane attack, it is essential that similar quantities of C5b-9 be present on cells despite variable extracellular Ca^{2+} concentrations. Because activation of the classical C pathway (by antibody) is Ca^{2+}-dependent, C5b-7 (which is not membranolytic) was initially formed in the presence of 0.5 mM extracellular Ca^{2+}. GEC were incubated with anti-Fx1A IgG (1 mg/ml) in modified Krebs-Henseleit buffer containing NaCl 145 mM, KCl 5 mM, $MgSO_4$ 0.5 mM, Na_2HPO_4 1 mM, $CaCl_2$ 0.5 mM, glucose 5 mM, and Hepes 20 mM, pH 7.40, for 40 minutes at 22°C. GEC were then washed and incubated with C8-deficient serum (C8DS, 10% vol/vol in Krebs-Henseleit buffer) for 40 minutes at 37°C to form C5b-7. C8DS was obtained from a patient with congenital absence of C8 activity [2]. After washing, C6-deficient rabbit serum (C6DS) was added as the source of C8 and C9 [3], at dilutions of 0.125 to 1.0% vol/vol, which resulted in final cell viabilities (see below) of 75 to 100% of control. The incubation was carried out at 37°C for 30 minutes, at which time the cytolytic endpoint was reached. Controls included antibody-sensitized GEC incubated with heat-inactivated human serum (56°C, 30 min) followed by the highest concentration of C6DS, and antibody-sensitized GEC incubated with C8DS followed by buffer alone.

In other experiments, GEC were first sensitized with anti-Fx1A, as above, and then washed and incubated for 40 minutes at 37°C with normal human serum (NHS) from a single individual. NHS was diluted in Krebs-Henseleit buffer to produce levels of cell viability that were 63 to 97% of control [5]. In control incubations, antibody was omitted during the first incubation and NHS was then employed at the highest concentration. Preliminary studies determined that viability of such cells was equivalent to the viability of cells incubated with antibody and heat-inactivated serum (that is, there was no antibody-independent cytotoxicity by C).

Measurements of cell viability

For these studies, GEC were plated into 24-well tissue culture dishes. Each measurement was performed in triplicate. Under normal culture conditions, viable GEC proliferate (that is, cell number increases over time), and this is associated with incorporation of ^{3}H-thymidine [29]. Thus, in the majority of experiments, cell viability was measured by incorporation of ^{3}H-thymidine (New England Nuclear, Boston, Massachusetts, USA and Mississauga, Ontario, Canada) for 24 hours after sensitization with antibody and incubation with C components [30]. Experiments were performed with subconfluent cells, two to three days after plating, as it was determined in preliminary studies that a zero-order ^{3}H-thymidine incorporation was present during the first three days after passage. The effects on viability of Ca^{2+} influx, Ca^{2+} mobilization, PKC activation, and eicosanoids were studied. Antibody-sensitized GEC were incubated with C8DS/C6DS or NHS (as described above and in **Results**), in the presence of vehicle or compounds including EGTA, 1,2,bis(o-aminophenoxy)ethane-N,N,-N',N'-tetraacetic acid (BAPTA, Molecular Probes, Eugene, Oregon, USA), phorbol myristate acetate (PMA, Sigma Chemical Co., St.

Louis, Missouri, USA), 1-(5-isoquinolinylsulfonyl)-2-methylpiperazine (H-7, Sigma), N-(6-aminohexyl)-1-naphthalenesulfonamide (W-7, Sigma), indomethacin (Sigma), or Ca^{2+} ionophore A23187 (Sigma). Control incubations (described above) included vehicle or added compound, and were performed in parallel. Stock solutions of BAPTA, PMA, H-7, W-7 and A23187 were prepared in dimethylsulfoxide, and indomethacin was in 0.2% sodium carbonate. Following incubation with C, cells were washed with buffer and pulsed for 24 hours with ^{3}H-thymidine (0.25 μCi/well) in culture medium. After 24 hours, cells were washed four times with saline and lysed with 0.1 N NaOH. The lysate was added to scintillation fluid and counted in a β-scintillation counter. Preliminary studies verified that ^{3}H in the lysate represented ^{3}H-thymidine incorporated into DNA; after incubation with ^{3}H-thymidine, some cells were incubated with cold thymidine for an additional three hours (to chase unincorporated ^{3}H-thymidine by competition), and were then washed and lysed with NaOH, as above. There were no significant differences between the radioactivities in NaOH lysates of cells that were chased with cold thymidine and cells that were not. Viability of GEC was calculated as E/C × 100 (%), where E is the ^{3}H-thymidine incorporation of C5b-9-treated GEC (plus compound or vehicle) and C is the ^{3}H-thymidine incorporation of control GEC (plus compound or vehicle) [30]. Values of ^{3}H-thymidine incorporation of control GEC were 10,000 to 40,000 cpm/well/24 hours.

In some experiments, viability was additionally determined by ^{3}H-thymidine retention [30]. GEC were prelabelled with ^{3}H-thymidine for 24 hours, as above. Cells were washed and were then incubated with antibody and C components (plus compound or vehicle), as above. After these incubations, cells were maintained in culture medium for 48 hours, at which time medium was collected, and NaOH was added to culture wells. The radioactivity in the culture medium at 48 hours (representing release of ^{3}H-thymidine from cells), and in the NaOH lysate of each well were measured. Specific ^{3}H-thymidine release of each sample was calculated as $[(R - S)/(M - S)] \times 100$, where R is experimental release, S is spontaneous release, and M is maximum release (R + NaOH-induced release) [5]. ^{3}H-thymidine retention was then calculated as [1-(E/C)] × 100 (%), where E is the ^{3}H-thymidine release of C5b-9-treated GEC (plus compound or vehicle) and C is the ^{3}H-thymidine release of control GEC (plus compound or vehicle). Preliminary experiments demonstrated that appearance of radioactivity in the culture medium at 48 hours represented release of ^{3}H-thymidine from DNA due to membranolysis; digitonin was added to prelabelled GEC to permeabilize plasma membranes [21]. Just after addition of digitonin, there was no increase in medium radioactivity, but after 48 hours the medium contained 97% of maximum releasable radioactivity. In cells not treated with digitonin (spontaneous release), <30% of maximum releasable radioactivity appeared in the medium.

Quantitation of C9 binding

To determine if treatments with BAPTA or PMA affected formation of C5b-9, the amount of assembled C5b-9 was assessed by the binding of ^{125}I-C9, as modified from Sims and Wiedmer [19]. Purified C9 (Cytotech, San Diego, California, USA) was iodinated to a specific activity of 9×10^5 cpm/μg using Iodobeads (Pierce Chemical Co., Rockford, Illinois, USA), according to the manufacturer. Using a C9 hemolytic assay [adapted from 2], it was determined that there was no loss of C9 activity following iodination. For binding studies, GEC were placed into suspension, and were then incubated with anti-Fx1A IgG (as above). After washing, aliquots of GEC (approximately 2.5×10^5 cells in 0.1 ml) were incubated with C9-deficient human serum (C9DS, Cytotech) or heat-inactivated serum (controls), diluted 10% vol/vol in buffer containing 0.5 mM Ca^{2+} for 40 minutes at 37°C. After washing, cells were loaded with 50 μM BAPTA or vehicle (in buffer containing no added Ca^{2+}) for 20 minutes, and ^{125}I-C9 (5.5 μg/ml, that is at 10% of normal serum concentration) was then added for a further 20 minutes. In experiments where the acute effect of PMA, and the prolonged effect of PMA (to deplete PKC) were assessed, ^{125}I-C9 was added together with C9DS or heat-inactivated serum (diluted 10% vol/vol in buffer containing 0.5 mM Ca^{2+}), and incubations were carried out as a single step. At the end of the incubations, bound ^{125}I-C9 was separated from free ^{125}I-C9 by filtration through glass-fiber filters that had been pretreated with polyethylenimine [31]. Specific binding of C9 was determined by subtracting the filter-associated radioactivity of GEC incubated with heat-inactivated serum and ^{125}I-C9 from that of cells incubated with C9DS and ^{125}I-C9.

Assessment of mitochondrial respiration

Mitochondrial respiratory activity of GEC was determined by measuring the rate of ATP production in the presence of an excess of ADP and substrates (glutamate and malate), according to a modification of the method of Harris et al [32]. GEC were plated into 12-multiwell dishes and experiments were performed with cells at confluence. GEC were first incubated with antibody, followed by 10% C8DS (as described above) and 1% C6DS (with and without Ca^{2+} ionophore A23187) for one hour at 37°C. Control incubations were carried out as described above. Cells were then washed and incubated in buffer containing KCl 120 mM, KH_2PO_4 5 mM, glutamate 5 mM, malate 5 mM, butyrate 1 mM, Hepes 10 mM and EGTA 2 mM, pH 7.40. Ouabain (0.1 mM), digitonin (0.09 mg/ml) and ADP (2.5 mM) were added sequentially to each well. After 10 minutes of incubation at 37°C, 0.1 ml of buffer was removed from each well, diluted in 1 ml Tris buffer (pH 8.2) and stored on ice for ATP assay. ATP was measured using the luciferase assay with purified luciferin-luciferase (Analytical Luminescence Laboratory, San Diego, California, USA) [33]. Under these conditions, the rate of ATP production was constant over the period measured, indicating an excess of substrates. Thus, this assay represents a measure of state III respiration.

Measurement of F-actin content

F-actin content was determined by quantitating the binding of nitrobenzo-oxadiazole (NBD)-phallacidin (Molecular Probes) [34, 35]. GEC were plated into 6-multiwell dishes and experiments were performed with cells at or near confluence. GEC were incubated with antibody and C8DS (as described above), or heat-inactivated serum in controls. After washing, cells were incubated with 1% C6DS at 37°C. At serial time intervals, cells were washed, fixed with 3.7% paraformaldehyde in phosphate buffered saline, permeabilized with 0.5% Triton X-100 (5 min, 22°C) and incubated with NBD-phallacidin (83 nM) for 20 minutes at 37°C. After three washes with phosphate buffered

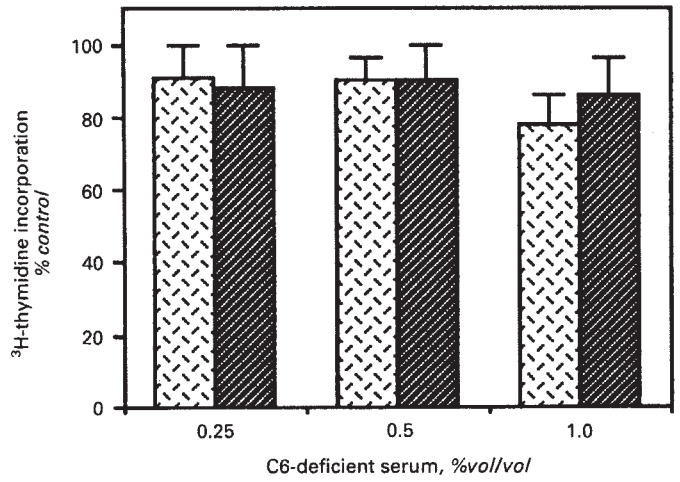

Fig. 1. *Effect of C5b-9-induced Ca^{2+} influx on ^{3}H-thymidine incorporation.* Antibody and C5b-7-sensitized GEC were incubated with C6DS (0.25 to 1.0% vol/vol) in buffer containing 0.5 mM Ca^{2+} with (▨) and without (▨) 4 mM EGTA. Control cells (**Methods**) were also incubated in buffer containing 0.5 mM Ca^{2+} with and without EGTA. GEC were then incubated with ^{3}H-thymidine in culture medium. ^{3}H-thymidine incorporation is presented as a ratio of incorporation in C5b-9-treated GEC with and without EGTA to that in control GEC with and without EGTA, respectively. Inhibition of Ca^{2+} influx with EGTA did not affect ^{3}H-thymidine incorporation. Values represent mean ± SEM of four experiments performed in triplicate. No statistically significant differences were found.

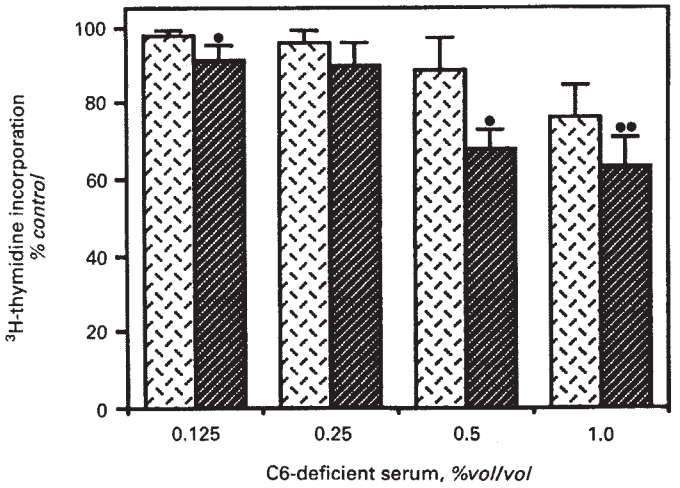

Fig. 2. *Effect of C5b-9-induced Ca^{2+} mobilization on ^{3}H-thymidine incorporation.* Antibody and C5b-7-sensitized GEC were incubated in buffer containing no added Ca^{2+} with (▨) and without (▨) BAPTA-AM (50 μM), followed by C6DS (0.125 to 1.0% vol/vol). Control cells (**Methods**) were also incubated in buffer containing no added Ca^{2+} with and without BAPTA-AM. Cells were then incubated with ^{3}H-thymidine in culture medium. ^{3}H-thymidine incorporation was reduced in the cells that had been loaded with BAPTA, suggesting that C5b-9-induced increase in $[Ca^{2+}]_i$ due to Ca^{2+} mobilization protects GEC from C-induced injury. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.025$ BAPTA vs. No added Ca^{2+}. Values represent mean ± SEM of four experiments performed in triplicate.

saline, the cytoskeleton-bound NBD-phallacidin was extracted with methanol for one hour at 37°C [35]. The fluorescence of the methanol extract (in arbitrary units) was then determined in a spectrofluorimeter (Perkin-Elmer, Norwalk, Connecticut, USA); excitation wavelength = 495 nm, emission wavelength = 510 nm). Results were expressed as a fluorescence ratio of C5b-9-treated cells to controls. GEC treated with Ca^{2+} ionophore A23187 were processed similarly to the above. In preliminary studies, it was determined that 83 nM NBD-phallacidin saturated all F-actin binding sites.

Statistics

Data are presented as means ± SEM. Comparisons were made by a two-tailed paired Student's *t*-test, except where indicated otherwise.

Results

Effect of $[Ca^{2+}]_i$ on C-mediated injury

The first series of experiments were designed to establish whether the MAC-induced increase in $[Ca^{2+}]_i$ affected GEC viability. Previously, we have demonstrated that addition of C8 and C9 to C5b-7-sensitized GEC results in an influx of Ca^{2+} [21]. Figure 1 demonstrates that chelation of extracellular Ca^{2+} with 4 mM EGTA just prior to addition of C6DS (0.25, 0.5 and 1.0% vol/vol, for 30 min) to C5b-7-sensitized GEC did not alter the subsequent 24 hour ^{3}H-thymidine incorporation, as compared to cells where C6DS was added in the presence of 0.5 mM Ca^{2+}. Thus, Ca^{2+} influx did not affect C-induced cytotoxicity. EGTA was not independently toxic to GEC, that is, ^{3}H-thymidine incorporation of control cells following EGTA treatment (30 min) was similar to that of control cells that were not treated with EGTA.

Influx of Ca^{2+} constitutes only part of the C5b-9-induced rise in $[Ca^{2+}]_i$, the remainder being due to mobilization of Ca^{2+} from intracellular stores [21]. To determine if MAC-induced Ca^{2+} mobilization affects ^{3}H-thymidine incorporation, prior to the addition of C6DS, C5b-7-sensitized GEC were loaded for 20 minutes at 37°C with the intracellular Ca^{2+} chelator, BAPTA (50 μM BAPTA-AM, in buffer without added Ca^{2+}), or with vehicle. C6DS (0.125 to 1.0% vol/vol in buffer without added Ca^{2+}) was then added with BAPTA or vehicle for 30 minutes. In cells where increases in $[Ca^{2+}]_i$ were buffered with BAPTA, the subsequent 24 hour ^{3}H-thymidine incorporation (expressed as a % of BAPTA-treated control cells) was decreased, as compared to cells where C6DS was added with vehicle alone (Fig. 2). To confirm these results, we also examined the effect of C5b-9 on cell viability by measuring ^{3}H-thymidine retention (that is, release of ^{3}H-thymidine from prelabeled cells into the medium after 48 hr) in the presence and absence of $[Ca^{2+}]_i$ buffering. The result of these experiments was similar to that of the experiments measuring ^{3}H-thymidine incorporation (Fig. 2). In the absence of BAPTA, ^{3}H-thymidine retention was 72 ± 11% of control at 0.25% C6DS and 78 ± 10% of control at 0.125% C6DS; in the presence of BAPTA, ^{3}H-thymidine retention declined to 58 ± 11% of control and 60 ± 12% of control, respectively ($P < 0.05$, $N = 4$ experiments). These data indicate that GEC injury is enhanced by buffering the Ca^{2+} mobilized by C5b-9, and suggest that C5b-9-induced Ca^{2+} mobilization protects GEC from C5b-9-induced injury. It should be recognized, however, that in addition to buffering the C-induced increase in

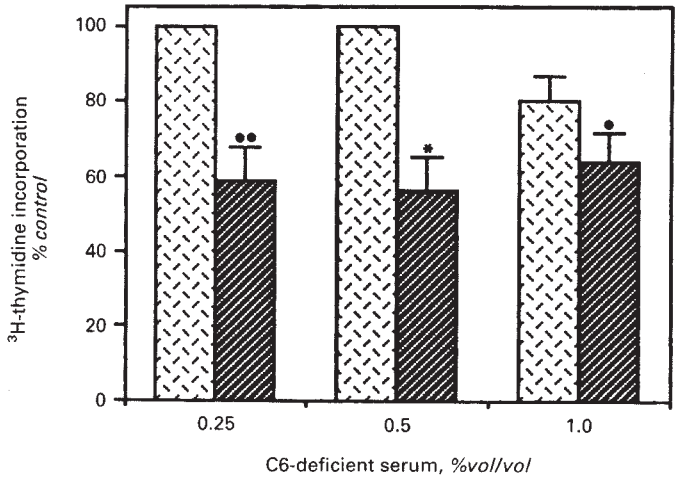

Fig. 3. *Effect of C5b-9 and Ca^{2+} ionophore A23187 on ^{3}H-thymidine incorporation.* Antibody and C5b-7-sensitized GEC were incubated with C6DS (0.25 to 1.0% vol/vol) in Ca^{2+}-containing buffer with and without Ca^{2+} ionophore (5 μM). Control cells (**Methods**) were also incubated with (▨) and without (▨) ionophore. Cells were then incubated with ^{3}H-thymidine. Formation of C5b-9 in the presence of ionophore reduced ^{3}H-thymidine incorporation. $^{\bullet}P < 0.05$, $^{\bullet\bullet}P < 0.025$, $^{*}P < 0.01$ A23187 vs. Vehicle. Values represent mean ± SEM of four experiments performed in triplicate.

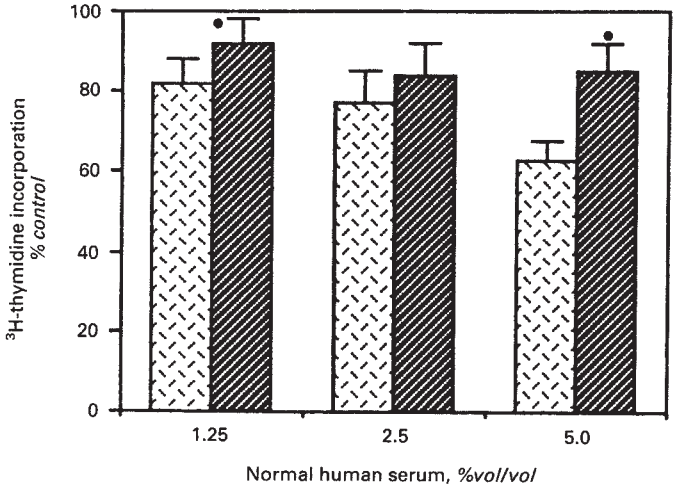

Fig. 4. *^{3}H-thymidine incorporation following protein kinase C (PKC) activation by PMA.* Antibody-sensitized GEC were incubated with NHS (1.25 to 5.0% vol/vol) with (▨) and without (▨) PMA (250 ng/ml). Control cells (**Methods**) were also incubated with and without PMA. Cells were then incubated with ^{3}H-thymidine. Addition of PMA enhanced ^{3}H-thymidine incorporation. $^{\bullet}P < 0.05$ PMA vs. Vehicle. Values represent mean ± SEM of four experiments performed in triplicate.

$[Ca^{2+}]_i$, BAPTA might have lowered basal $[Ca^{2+}]_i$, which possibly may have also contributed to the enhancement of GEC injury in the presence of BAPTA.

As with EGTA, BAPTA did not affect the ^{3}H-thymidine incorporation of control GEC, suggesting that it was not independently toxic. However, further experiments were carried out to confirm that the reduction in ^{3}H-thymidine incorporation in the presence of BAPTA (Fig. 2) was due to buffering of increases in $[Ca^{2+}]_i$, and was not due to independent toxicity. Antibody-sensitized GEC were incubated for 40 minutes with NHS at concentrations of 1.25, 2.5 and 5.0% vol/vol in buffer containing 0.5 mM Ca^{2+}, with BAPTA-AM (50 μM) or with vehicle ($N = 3$ experiments). (When BAPTA-AM is added to cells in buffer containing an extracellular Ca^{2+} concentration near physiological, such as 0.5 mM, it will enter cells complexed with Ca^{2+}, and, therefore, the $[Ca^{2+}]_i$ buffering capacity of BAPTA will be diminished. It should be noted that BAPTA-AM was added to buffer containing no added Ca^{2+} in the experiments shown in Fig. 2.) After incubation with NHS, cells were washed and incubated with ^{3}H-thymidine. Unlike Figure 2, in the presence of 0.5 mM extracellular Ca^{2+}, there were no significant differences in ^{3}H-thymidine incorporation between BAPTA- and vehicle-treated GEC at each concentration of NHS, indicating that BAPTA was not independently toxic. (The data for these experiments are not presented, as they are similar to the results of the vehicle-treated cells shown in Fig. 4, below.)

In contrast to results shown in Figure 2, when large increases in $[Ca^{2+}]_i$ were associated with MAC formation, there was enhanced cytotoxicity. C6DS was added to C5b-7-sensitized (and to control) GEC in the presence of the Ca^{2+} ionophore A23187 (5 μM) for 30 minutes. Subsequent 24 hour ^{3}H-thymidine incorporation (expressed as a % of Ca^{2+} ionophore-treated control cells) was decreased below that of cells where C6DS had been added in the presence of vehicle alone (Fig. 3). Ca^{2+} ionophore by itself was modestly toxic, that is, ^{3}H-thymidine incorporation of ionophore-treated control GEC was 75% of vehicle-treated controls.

Effect of protein kinase C (PKC) activation on C-mediated injury

Several of the MAC-induced products of phospholipase activation [21] are potential activators of PKC [22]. To determine if enhancement of PKC activation affects C5b-9-induced GEC injury, antibody-sensitized cells were incubated for 40 minutes with NHS at concentrations of 1.25 to 5.0% vol/vol, with PMA (250 ng/ml) or with vehicle. In cells where PMA had been added to NHS, the subsequent 24 hour ^{3}H-thymidine incorporation (expressed as a % of PMA-treated control cells) increased above that of cells incubated with NHS and vehicle (Fig. 4). Thus, activation of PKC by PMA decreased C5b-9-mediated injury. There was no difference in ^{3}H-thymidine incorporation between control cells treated with PMA and control cells treated with vehicle. To confirm that increased ^{3}H-thymidine incorporation with PMA was due to less C-induced cytolysis, we prelabelled GEC with ^{3}H-thymidine for 24 hours, and examined the effect of C5b-9 and PMA on ^{3}H-thymidine retention. Similarly to the results shown in Figure 4, ^{3}H-thymidine retention was increased in cells incubated with C and PMA as compared to C and vehicle. In the absence of PMA, ^{3}H-thymidine retention was 86 ± 4% of control (1.25% NHS), 84 ± 4% (2.5% NHS), and 65 ± 14% (5.0% NHS); in the presence of PMA, ^{3}H-thymidine retention was 97 ± 2% ($P < 0.03$), 95 ± 3% ($P < 0.005$), and 72 ± 11%, respectively ($N = 5$ experiments).

Two approaches were used to determine if C5b-9 might activate PKC, leading to modulation of C-mediated injury. First, C-induced injury was measured in GEC that were prein-

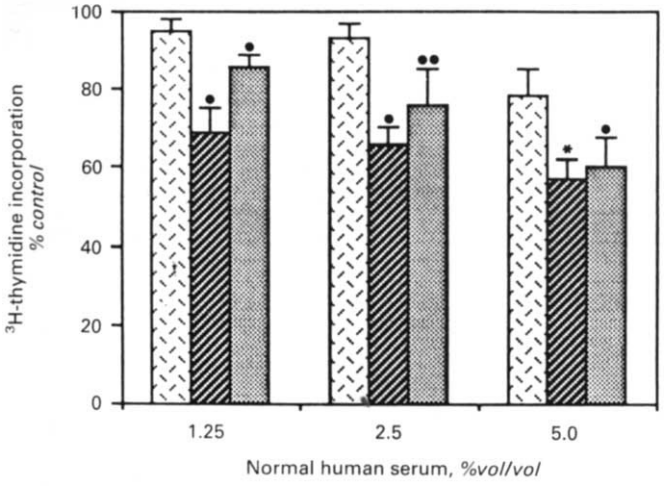

Fig. 5. *C-mediated injury following protein kinase C (PKC) down-regulation, or inhibition with H-7.* GEC were incubated with antibody and NHS (1.25 to 5.0% vol/vol). One group of GEC was untreated (▨), a second group had been depleted of PKC by an 18 hr preincubation with PMA (▨, 1.5 μg/ml), and in a third group, PKC was inhibited with H-7 (▨, 25 μM). Control incubations (**Methods**) also included PKC-depleted and H-7-treated GEC (▨). Cells were then incubated with ^{3}H-thymidine. ^{3}H-thymidine incorporation was reduced in PKC-depleted cells, and in H-7-treated cells, indicating that C5b-9-induced activation of PKC protects GEC from C-mediated injury. $^*P < 0.05$, $^{**}P < 0.03$, $^•P < 0.01$ PKC-depleted or H-7 vs. Untreated. Values represent mean ± SEM of four experiments performed in triplicate.

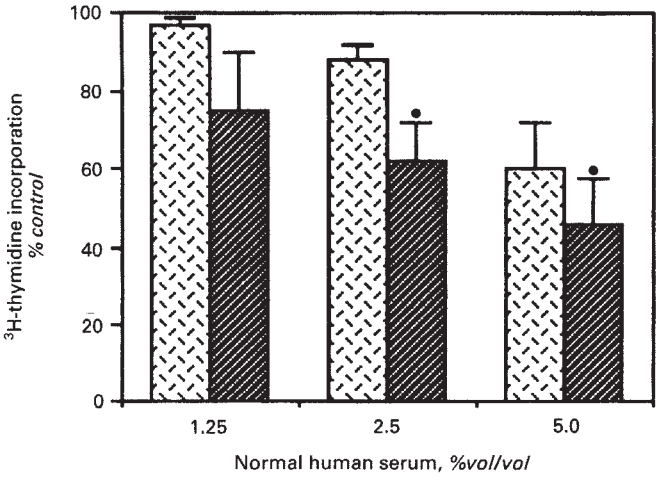

Fig. 6. *Effect of indomethacin on ^{3}H-thymidine incorporation.* Antibody-sensitized GEC were incubated with NHS (1.25 to 5.0% vol/vol) with (▨) and without (▨) indomethacin (5 μM). Control cells (**Methods**) were also incubated with and without indomethacin. Cells were then incubated with ^{3}H-thymidine. Addition of indomethacin reduced ^{3}H-thymidine incorporation of C5b-9-treated cells. $^•P < 0.025$ Indomethacin vs. Vehicle. Values represent mean ± SEM of four experiments performed in triplicate.

cubated for 18 hours with a high dose of PMA (1.5 μg/ml) to down-regulate PKC [36]. When these PKC-depleted cells were then incubated with antibody and NHS, the subsequent ^{3}H-thymidine incorporation (expressed as a % of PKC-depleted control cells) was markedly reduced below that of C5b-9-treated GEC that were not depleted of PKC (Fig. 5). ^{3}H-thymidine incorporation of PKC-depleted control cells was not significantly different from PKC-replete control cells. In a second series of experiments, antibody-sensitized GEC were incubated with NHS, in the presence of vehicle or the protein kinase inhibitor H-7 [37]. As seen with PKC down-regulation, inhibition of PKC with H-7 (25 μM) decreased the subsequent ^{3}H-thymidine incorporation (Fig. 5), although the effect was less pronounced. Finally, because PMA and H-7 are lipophilic compounds that could potentially contribute to nonspecific membranolytic effects, we also examined if C-mediated injury was affected by W-7 [38], a compound similar in chemical structure to H-7, but a weak inhibitor of protein kinases (W-7 inhibits calmodulin). In the presence of W-7 (25 μM, $N = 4$ experiments), ^{3}H-thymidine incorporation was 90 ± 5% of control (1.25% NHS), 84 ± 6% (2.5% NHS) and 71 ± 5% (5.0% NHS). These values were not different from vehicle-treated cells: 88 ± 6%, 80 ± 7% and 71 ± 8%, respectively, indicating that the effect observed with H-7 was unlikely to be due to nonspecific injury. Thus, inability of C5b-9 to activate PKC (due to PKC depletion), or inhibition of PKC with H-7 during C attack, both enhance C-mediated injury, while exogenous activation of PKC by PMA reduces injury.

If prolonged preincubation of GEC with a high concentration of PMA resulted in PKC depletion, then it would be expected that in such cells, there would be no reduction in injury when C5b-9 is formed in the presence of PMA, that is, the effect of PMA shown in Figure 4 would be abolished. After 18 hours of preincubation with PMA (1.5 μg/ml), GEC were incubated with antibody, followed by NHS (5.0% vol/vol) with and without PMA (250 ng/ml; as in Fig. 4). Unlike the results in Figure 4, there was no difference in subsequent ^{3}H-thymidine incorporation (expressed as % control) between NHS + PMA and NHS without PMA (57 ± 8% in both groups; $N = 3$ experiments). Moreover, if the addition of H-7 (25 μM) to GEC inhibited PKC, in these cells the effect of PMA on C-induced injury (shown in Fig. 4) should be abolished. GEC were incubated in the presence of H-7 (25 μM) with antibody, followed by NHS (5.0% vol/vol) with and without PMA (250 ng/ml). In contrast to Figure 4, there was no difference in subsequent ^{3}H-thymidine incorporation between the two groups (PMA: 41 ± 4% vs. no PMA: 49 ± 1%, $N = 3$ experiments).

To determine if production of eicosanoids might be involved in modulating C-mediated GEC injury, ^{3}H-thymidine incorporation was measured in antibody-sensitized GEC that were incubated with NHS with and without indomethacin (5 μM). Figure 6 demonstrates that cyclooxygenase inhibition by indomethacin reduced ^{3}H-thymidine incorporation in GEC. Control cells with and without indomethacin demonstrated similar ^{3}H-thymidine incorporation, indicating that indomethacin was not independently cytotoxic.

Quantitation of C9 binding

Table 1 demonstrates that formation of C5b-9 (assessed by the binding of ^{125}I-C9) was not altered after cells were loaded with BAPTA (50 μM), or in the presence of PMA (250 ng/ml). Similarly, treatment of GEC with PMA (1.5 μg/ml) for 18 hours to deplete PKC did not affect ^{125}I-C9 binding. These results indicate that the modulation of C5b-9-induced injury by the above treatments is not due to quantitative differences in C5b-9 formation.

Table 1. Quantitation of C5b-9 formation

	Specific binding of ^{125}I-C9 *ng/10^6 cells*	*N*
A Vehicle	4.29 ± 1.44	3
BAPTA-AM *50 μM*	4.45 ± 1.91	3
B Vehicle	5.72 ± 2.19	3
PMA *250 ng/ml*	6.43 ± 2.60	3
C PKC-replete *untreated*	8.15 ± 1.75	4
PKC-depleted *PMA, 1.5 μg/ml*	6.19 ± 2.03	4

A. Antibody-sensitized GEC were incubated with C9DS (10% vol/vol in buffer containing 0.5 mM Ca^{2+}), followed by BAPTA-AM or vehicle (in buffer with no added Ca^{2+}), and the ^{125}I-C9 (**Methods**). B. Antibody-sensitized GEC were incubated with C9DS that was reconstituted with ^{125}I-C9 (10% vol/vol in buffer containing 0.5 mM Ca^{2+}), in the presence of PMA or vehicle. C. PKC-replete and PKC-depleted GEC (18 hr preincubation with PMA, 1.5 μg/ml) were sensitized with antibody, and were incubated with C9-DS that was reconstituted with ^{125}I-C9 (10% vol/vol in buffer containing 0.5 mM Ca^{2+}). In control incubations, antibody-sensitized GEC were incubated with heat-inactivated serum supplemented with ^{125}I-C9. Each incubation was performed in duplicate or triplicate. Values are mean ± SEM, *N* indicates number of experiments. There are no statistically significant differences between groups.

Table 2. Effect of C5b-9 and Ca^{2+} ionophore A23187 on ATP generation by GEC

	ATP *nM/well*	*N*
No A23187		
Control	36 ± 3	28
C5b-9	34 ± 3	14
A23187		
Control	21 ± 2	28
C5b-9	19 ± 2	14

ATP generation was determined in digitonin-permeabilized GEC that were incubated with C5b-9 (and in control cells) in the presence and absence of A23187 (5 μM). Values are mean ± SEM. A statistically significant difference was present between groups ($P < 0.0001$, one-way analysis of variance). $P < 0.0001$ Control (no A23187) vs. Control (+A23187).

Effect of C5b-9 on mitochondrial function

The above studies have shown that an increase in $[Ca^{2+}]_i$ protects GEC from C-mediated injury, as measured by ^{3}H-thymidine incorporation and release. We then investigated if C5b-9-mediated rise in $[Ca^{2+}]_i$ could be detrimental to other cellular functions, for example, in generation of ATP by mitochondria. GEC were incubated with C5b-9, and then following permeabilization with digitonin, we measured the ability of GEC to synthesize ATP in the presence of EGTA, ADP and substrates. Table 2 demonstrates that C5b-9 did not impair ATP generation, indicating the increase in $[Ca^{2+}]_i$ due to C5b-9 is not associated with impaired state III mitochondrial respiration. Since addition of the Ca^{2+} ionophore A23187 with C5b-9 markedly enhanced C5b-9-mediated cytotoxicity (Fig. 3), the effect on ATP generation of C5b-9 together with Ca^{2+} ionophore was also examined. Ionophore alone impaired ATP generation, but there did not appear to be an additive effect of ionophore and C5b-9 (Table 2).

Effect of C5b-9 on F-actin content

Hydrolysis of inositol phospholipids and changes in $[Ca^{2+}]_i$ may affect the structure of the cytoskeleton [34, 39]. To determine if C5b-9-induced changes in plasma membrane morphology might be due to alterations in the cytoskeleton, the F-actin content (NBD-phallacidin binding) was determined in GEC incubated with C5b-9, and in controls. In addition, the effect of increased $[Ca^{2+}]_i$ was examined by incubating GEC with the Ca^{2+} ionophore A23187 (5 μM). As demonstrated in Figure 7, neither C5b-9 nor Ca^{2+} ionophore significantly altered the F-actin content of GEC.

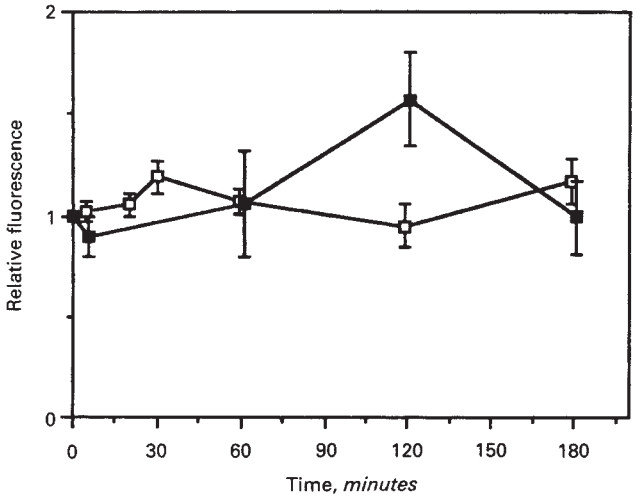

Fig. 7. *Effect of C5b-9 and Ca^{2+} ionophore A23187 on F-actin content of GEC.* Binding of NBD-phallacidin (Methods) was determined at serial intervals in GEC incubated with C5b-9 (–□–) and with A23187 (–■–, 5 μM). Values are presented as the ratio of NBD-phallacidin fluorescence in C5b-9-treated and A23187-treated GEC to respective controls (mean ± SEM of 3 to 6 determinations at each time point). There are no significant differences in fluorescence between treated cells and controls.

Discussion

Several intracellular pathways can be activated during C attack of nucleated cells [17–21], some of which can downmodulate, and others, enhance the membranolytic effect of C5b-9 [7–13]. Rat membranous nephropathy is an example of the pathophysiological consequences of nonlytic cellular injury by C5b-9. Insertion of C5b-9 into the GEC plasma membranes results in membrane vesiculation and foot process effacement, and is associated with loss of glomerular capillary wall permselectivity [2]. To understand the pathogenesis of morphological changes in rat membranous nephropathy, we recently examined changes in $[Ca^{2+}]_i$ and lipid mediators following formation of C5b-9 in cultured GEC, and we found that C5b-9 led to the activation of phospholipases (resulting in increased 1,2-DAG and free arachidonic acid), as well as an increase in $[Ca^{2+}]_i$ [21]. In the present study, we examined how these changes might affect GEC injury.

We observed that a MAC-induced increase in $[Ca^{2+}]_i$ may protect cells from C-mediated cytolysis, as buffering of $[Ca^{2+}]_i$ with BAPTA during formation of C5b-9 diminished cell viability (Fig. 2). Our results are similar to those reported previously in neutrophils [9] and Ehrlich cells [10, 11], with one exception. Whereas lowering of extracellular Ca^{2+} was sufficient to increase cytolysis in neutrophils and Ehrlich cells [9, 10], in our

study buffering of $[Ca^{2+}]_i$ (with BAPTA) was required to demonstrate the protective effect of an increase in $[Ca^{2+}]_i$, and abolition of only Ca^{2+} influx (with EGTA) without buffering Ca^{2+} mobilization was ineffective in reducing C lysis (Fig. 1). Addition of PMA to GEC during formation of the MAC also diminished C lysis (Fig. 4). Conversely, C lysis was enhanced in GEC that were preincubated with a high dose of PMA to down-regulate PKC, and in GEC that were treated with the PKC inhibitor H-7 (Fig. 5). These findings are consistent with MAC-induced activation of PKC, possibly resulting from increased DAG, $[Ca^{2+}]_i$, or free arachidonic acid [21], and indicate that PKC may protect GEC from C-induced injury. Activation of PKC by C5b-9 has been demonstrated in platelets [20] but, to our knowledge, the protective effect of PKC on cytolysis has not been previously shown. The mechanisms by which a rise in $[Ca^{2+}]_i$ and PKC activation reduce the amount of C-mediated injury remain undefined, although Ca^{2+} and PKC do not act by decreasing the efficiency of C5b-9 assembly (Table 1). Therefore, in further studies it will be necessary to examine the effect of Ca^{2+} and PKC on potential mechanisms of protection from C-mediated injury, such as stimulation of exocytosis of C5b-9 complexes from the plasma membranes, accelerated repair of the plasma membrane due to enhanced lipid or protein synthesis, or enhanced activity of Na^+/K^+ ATPases [8–13]. It should be noted that the recent ultrastructural demonstration of C5b-9-containing membrane vesicles in the urinary space in rat membranous nephropathy is consistent with exocytosis of C5b-9 complexes [4].

We previously observed that in GEC, cyclooxygenase inhibition with indomethacin partially decreased the C5b-9-induced rise in DAG and arachidonic acid, and that the prostaglandin H_2/thromboxane A_2 analogue U46619 could independently increase arachidonic acid and DAG [21]. Thus, production of eicosanoids from C-induced arachidonate release can secondarily stimulate production of DAG and arachidonate, which may account for the augmented C-mediated cytotoxicity in response to cyclooxygenase inhibition with indomethacin (Fig. 6). By abolishing the amplification effect of eicosanoids on DAG or free fatty acid production, cyclooxygenase inhibition might have led to less PKC activation and, consequently, less of a protective effect.

In the experiments described above, we observed that C lysis was diminished by C-induced phospholipase activation and the associated increase in $[Ca^{2+}]_i$. However, it has been proposed that increased $[Ca^{2+}]_i$ could impair mitochondrial respiration [23] and, therefore, we sought evidence of sublytic mitochondrial dysfunction. We found that C attack did not impair ATP generation by mitochondria (Table 2), indicating that the relatively small increase in $[Ca^{2+}]_i$ induced by C5b-9 [21] was not toxic to mitochondria, and consistent with observations that mitochondria can buffer large amounts of Ca^{2+} without impairment in oxidative phosphorylation [23]. However, a large increase in $[Ca^{2+}]_i$, produced by the Ca^{2+} ionophore A23187, was associated with an impairment in ATP generation (Table 2). C5b-9-mediated cytotoxicity was aggravated by Ca^{2+} ionophore (Fig. 3), but C5b-9 did not modulate the Ca^{2+} ionophore-induced reduction in ATP generation (Table 2). These results are consistent with the view that intact mitochondrial activity is necessary for cells to withstand C attack. Following the activation of phospholipases by C5b-9, there is release of free fatty acids [21] and, probably, formation of lysophospholipids. The integrity of the plasma membrane may be partly dependent on reacylation of lysophospholipids or de novo phospholipid synthesis, processes that appear to be impaired in the presence of ATP depletion [40]. Thus, mitochondrial dysfunction may prevent maintenance of membrane integrity during C attack.

It is now apparent that activation of phospholipase C (such as, by chemotactic peptide f-met-leu-phe in neutrophils), an increase in $[Ca^{2+}]_i$, activation of PKC (by the addition of PMA), and hydrolysis of phosphatidylinositol bisphosphate can lead to a change in cytoskeletal structure, in particular, the content of F-actin [25, 35, 39]. Because alterations in plasma membrane morphology (such as those observed in rat membranous nephropathy) could potentially result from underlying changes in the cytoskeleton, and because, in GEC, C5b-9 increases $[Ca^{2+}]_i$ and activates phospholipase C (resulting in hydrolysis of phosphatidylinositol bisphosphate) we investigated if C5b-9 could affect the amount of F-actin. Our results demonstrated that F-actin was not altered by C5b-9 (Fig. 7), and suggest that the GEC morphological changes seen in rat membranous nephropathy are not associated with alterations in F-actin content. Larger increases in $[Ca^{2+}]_i$, induced by Ca^{2+} ionophore, also failed to alter the amount of F-actin. While these findings in GEC differ from cytoskeletal changes described in neutrophils following phospholipase C activation [35], it should be recognized that the latter are motile cells and, consequently, responses of the cytoskeleton may be much more pronounced. Also, these findings do not exclude possible qualitative changes in the cytoskeleton.

On the basis of this study and of previous observations of C-induced injury of nucleated cells, it can be proposed that C-induced GEC injury in rat membranous nephropathy is reduced by a rise in $[Ca^{2+}]_i$, by products of phospholipase activation, and by PKC. Several mechanisms may be involved in the protection of GEC from C attack, including the elimination of C5b-9 complexes from the plasma membrane by exocytosis [8]. Moreover, exocytosis of C5b-9 may be responsible for the plasma membrane vesiculation seen in rat membranous nephropathy [2, 4], as in cultured GEC [6]. Additional studies are required to elucidate the cellular mechanisms of proteinuria and other morphological changes, such as foot process effacement.

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