PKC-ζ is required in EGF protection of microtubules and intestinal barrier integrity against oxidant injury

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Banan, A., J. Z. Fields, D. A. Talmage, L. Zhang, and A. Keshavarzian. PKC-ζ is required in EGF protection of microtubules and intestinal barrier integrity against oxidant injury. Am J Physiol Gastrointest Liver Physiol 282: G794–G808, 2002. First published January 9, 2002; 10.1152/ajpgi.00284.2001.—Using monolayers of human intestinal (Caco-2) cells, we showed that epidermal growth factor (EGF) protects intestinal barrier integrity against oxidant injury by protecting the microtubules and that protein kinase C (PKC) is required. Because atypical PKC-ζ is abundant in wild-type (WT) Caco-2 cells, we hypothesized that PKC-ζ mediates, at least in part, EGF protection. Intestinal cells (Caco-2 or HT-29) were transfected to stably over- or under-express PKC-ζ. These clones were preincubated with low or high doses of EGF or a PKC activator [1-oleoyl-2-acetyl-sn-glycerol (OAG)] before oxidant (0.5 mM H2O2). Relative to WT cells exposed to oxidant, only monolayers of transfected cells overexpressing PKC-ζ (2.9-fold) were protected against oxidant injury as indicated by increases in polymerized tubulin and decreases in monomeric tubulin, enhancement of architectural stability of the microtubule cytoskeleton, and increases in monolayer barrier integrity toward control levels (62% less leakiness). Overexpression-induced protection was OAG independent and even EGF independent, but EGF significantly potentiated PKC-ζ protection. Most overexpressed PKC-ζ (92%) resided in membrane and cytoskeletal fractions, indicating constitutive activation of PKC-ζ. Stably inhibiting PKC-ζ expression (95%) with antisense transfection substantially attenuated EGF protection as demonstrated by reduced tubulin assembly and increased microtubule disassembly, disruption of the microtubule cytoskeleton, and loss of monolayer barrier integrity. We conclude that 1) activation of PKC-ζ is necessary for EGF-induced protection, 2) PKC-ζ appears to be an endogenous stabilizer of the microtubule cytoskeleton and of intestinal barrier function against oxidative injury, and 3) we have identified a novel biological function (protection) among the atypical isoforms of PKC.

cytoskeleton; growth factors; epidermal growth factor; Caco-2 cells; gut barrier; protection; transfection; protein kinase C isoforms; inflammatory bowel disease

A FUNDAMENTAL PROPERTY of epithelial cells of the gastrointestinal (GI) tract is to function as a highly selective permeability barrier, permitting the absorption from the lumen of nutrients, water, and electrolytes but restricting passage of harmful proinflammatory and toxic molecules (e.g., immunoreactive antigens, endotoxin) into the mucosa or the systemic circulation. Loss of mucosal barrier integrity has been implicated in the pathogenesis of multiple organ system dysfunction, inflammatory bowel disease (IBD), necrotizing enterocolitis, ethanol- and nonsteroidal anti-inflammatory drug (NSAID)-induced chemical injury, and a variety of other GI disorders as well as several systemic disorders (e.g., alcoholic liver disease) (5, 30, 37). Pathogenesis of mucosal barrier dysfunction in these disorders remains poorly understood, but several studies, including our own (5–7, 9, 15, 17, 37), have shown that chronic gut inflammation is associated with high levels of reactive oxygen metabolites and that oxidants appear to be a key underlying cause of injury (31, 39, 42, 59). Oxidative injury is of clinical importance not only because reactive oxygen metabolites are common in inflammation but also because they can cause mucosal barrier hyperpermeability and, in turn, lead to the initiation and/or perpetuation of mucosal inflammation and injury (30, 31, 37, 38). For example, increases in epithelial barrier permeability after the injection of bacterial endotoxin into the mucosa in animal models can initiate an oxidative and inflammatory condition similar to IBD (59). Similarly, genetically engineered mice with a leaky gut develop intestinal inflammation (29).

We have been investigating endogenous protective mechanisms (e.g., growth factor signaling) against oxidant-induced barrier dysfunction in an effort to develop a rational basis for more effective treatment regimens for inflammatory disorders of the GI tract. We recently showed, using monolayers of human intestinal cells (Caco-2) as a model of barrier function, that epidermal growth factor (EGF) or transforming growth factor-α protect intestinal barrier integrity by stabilizing the microtubule cytoskeleton (5, 6, 8–11) in large part through the activation of protein kinase C (PKC) (8, 10, 11). Because involvement in protective mechanisms by PKC was a novel finding, we surmised that
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One or more specific isoforms of PKC might mediate the protective actions of PKC.

PKC consists of a family of serine and threonine-specific kinases. The PKC family, which includes at least 12 known isoenzymes, can be classified into three subfamilies on the basis of differences in sequence homology and cofactor requirement (2, 4, 17, 18, 25, 32, 34, 41, 44, 46, 48, 49, 50, 51, 53, 57, 60, 61). The conventional (or classic) PKC isoforms (α, β1, β2, γ) require calcium, diacylglycerol (DAG), and phospholipid for their activation, whereas the novel PKC isoenzymes (δ, ε, θ, η, μ) are calcium independent but require DAG and phospholipid. Activation of the third group, atypical PKC isoforms (λ, τ, ζ), is independent of both calcium and DAG (21). Intestinal epithelial cells, including Caco-2 cells, express at least five of these isoforms: PKC-α, PKC-β1, PKC-β2, PKC-δ, and PKC-ζ (1, 8, 11, 16, 21, 43, 53, 58). These isoforms differ in their activation, tissue expression, intracellular distribution, and substrate specificity, suggesting that each isoform has a unique, nonredundant role in signal transduction (1, 16, 32, 41, 44, 49, 50).

We (8) previously showed in wild-type Caco-2 intestinal cells that EGF induces the membrane translocation of native PKC-β1 and PKC-ζ isoforms, and therefore we considered each as a possible contributor to EGF-affected protection. Using transfected cells that either stably overexpressed PKC-β1 or could not express PKC-β1, we recently found (6, 8, 11) that PKC-β1, a conventional or classic, DAG-dependent isoform of PKC, was necessary for a substantial fraction, but not all, of EGF protection. We noted that protection mediated by PKC-β1 was DAG dependent because neither PKC-β1 overexpression nor low doses of 1-oleoyl-2-acetyl-sn-glycerol (OAG) alone afforded protection but together they led to protection. In the current report, we have explored the role of the ζ isoform of PKC because: 1) it is translocated to the membranes in wild-type Caco-2 cells by EGF; 2) unlike PKC-β1, it is an “atypical” PKC isoform; 3) it is of clinical and biological importance to more fully establish the idea that specific isoforms of PKC play fundamental roles in endogenous protective mechanisms of cells; and 4) a better understanding of the pathophysiology of hyperpermeability of the intestinal barrier and its prevention could lead to the development of novel therapeutics for inflammatory diseases of the GI tract related to oxidative injury.

Accordingly, we studied the PKC-ζ isoform utilizing targeted molecular interventions (transfection) that enabled us to develop two novel and stably transfected intestinal cell lines. In one, the atypical isoform PKC-ζ was reliably overexpressed; in the other, PKC-ζ expression was almost completely inhibited. Using these new models, we tested the hypothesis that EGF-induced protection against oxidant injury to both the microtubule cytoskeleton and intestinal barrier depends on activation of the ζ isoform but without the requirement for DAG (OAG).

Materials and Methods

Cell culture. Both Caco-2 and HT-29 cells were chosen because they form monolayers that morphologically resemble small intestinal cells, with defined apical brush borders, junctional complexes, and a highly organized microtubule network. Utility and characterization of these cell lines have been previously reported (4, 13, 24, 45).

Plasmids and stable transfection. Sense and antisense plasmids of PKC-ζ were constructed and then stably transfected as we previously described (10, 11, 21). Expression was controlled by β-actin promoter. The antisense PKC-ζ plasmid (β-actin SP72-As-PKC-ζ) was constructed by ligating the 2.3-kb EcoRI fragment of PKC-ζ cDNA from pGK-PKC-ζ (21) into the unique EcoRI sites of the β-actin SP72 vector. The antisense orientation of the plasmid was confirmed by SamI restriction digestion (21).

After transfection by Lipofectin reagent (GIBCO-BRL), cells were subjected to G418 selection (0.6 mg/ml) over 4 wk. Resistant cells were maintained in culture media/fetal bovine serum and 0.2 mg/ml G-418 (selection medium). PKC protein expression or lack of it was verified by Western blot analysis of cell lysates (see Fractionation and Western immunoblotting of PKC). Multiple clones stably overexpressing PKC-ζ or lacking PKC-ζ were assessed by immunoblotting and plated on transwell cell culture inserts, allowed to form confluent monolayers, and subsequently used for experiments.

Experimental design. First, postconfluent monolayers of wild-type cells were preincubated with EGF (1 or 10 ng/ml) or isotonic saline for 10 min and were then exposed to oxidant (0.5 mM H2O2) or vehicle (saline) for 30 min. As we have previously shown (5–7, 11, 13, 15), H2O2 at 0.5 mM disrupts microtubules and barrier integrity; EGF at 10 ng/ml (but not 1 ng/ml) prevents this disruption. These experiments were then repeated using cell monolayers either stably overexpressing or almost completely lacking PKC-ζ. Reagents were applied on the apical side of monolayers unless otherwise indicated. In all experiments, barrier function, microtubule cytoskeletal stability (cytoarchitecture, tubulin assembly/disassembly), and PKC-ζ subcellular distribution were then assessed.

Second, cell monolayers stably overexpressing PKC-ζ were preincubated (10 min) with EGF (1 or 10 ng/ml) or vehicle before exposure (30 min) to damaging concentrations of oxidant (0.5 mM H2O2) or vehicle (8).

Third, monolayers of antisense-transfected cells stably lacking PKC-ζ protein expression were treated with high (protective) doses of EGF and then oxidant. Expression levels of PKC-ζ were determined by immunoblotting. In a corollary series of experiments, we investigated the effects of PKC-ζ over- or underexpression on the state of tubulin assembly and disassembly and on stability of the cytoarchitecture of the microtubule cytoskeleton. Monomeric and polymerized fractions of tubulin (the structural protein subunit of microtubules) were isolated and then analyzed by quantitative immunoblotting (5, 6, 8, 13). Microtubule integrity was assessed by 1) immunofluorescent labeling and fluorescence microscopy to determine the percentage of cells with normal microtubules, 2) detailed analysis by high-resolution laser scanning confocal microscopy (LSCM), and 3) quantitative immunoblot analysis of monomeric (S1) and polymerized (S2) tubulin fractions.

Fractionation and Western immunoblotting of PKC. Differentiated cell monolayers grown in 75-cm² flasks were processed for the isolation of the cytosolic, membrane, and cytoskeletal fractions as previously described by others and by us (1, 8, 11). Protein content of the various cell fractions was
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assessed by the Bradford method (19). For total PKC extraction, scraped monolayers were placed directly into 1.5 ml of a standard cold lysis buffer (4°C) and subsequently ultracentrifuged. Supernatant was used for bulk protein determination.

For immunoblotting, samples (75 µg protein/lanes) were added to SDS buffer (250 mM Tris·HCl, pH 6.8, 2% glycerol, 5% mercaptoethanol), boiled for 5 min, and then separated on 7.5% SDS-PAGE (8, 11). The immunoblotted proteins were incubated for 2 h in TWEEN 20, Tris-buffered saline, 1% BSA, and the primary mouse monoclonal anti-PKC-ζ (Santa Cruz Biotech, Santa Cruz, CA) at 1:2,000 dilution for 1 h at room temperature. A horseradish peroxidase-conjugated goat anti-mouse antibody (Molecular Probes, Eugene, OR) was used as a secondary antibody at 1:4,000 dilution. Proteins on membranes were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and autoradiography and subsequently analyzed by densitometry. In preliminary studies using total PKC extracts, we confirmed that overexpression of PKC-ζ or antisense inhibition of PKC-ζ expression did not affect the relative expression levels of other PKC isoforms nor did it injure the cell monolayer barrier.

Immunofluorescent staining and high-resolution LSCM of microtubules. Cells from monolayers were fixed in cytoskeletal stabilization buffer and then postfixed in 95% ethanol at −20°C as we previously described (5, 6, 8, 12–14). Cells were subsequently processed for incubation with a primary antibody, monoclonal mouse anti-β-tubulin (Sigma, St. Louis, MO) at 1:200 dilution for 1 h at 37°C, and were then incubated with a secondary antibody (FITC-conjugated goat anti-mouse; Sigma) at 1:50 dilution for 1 h at room temperature. Slides were washed three times in T-PBS and subsequently mounted in aquamount. After being stained, cells were observed with an argon laser (λ = 488 nm) using a ×63 oil immersion plan-apochromat objective, 1.4 numerical aperture (Zeiss). Single cells and/or a clump of two to three cells from desired areas of monolayers were processed using the image processing software on a Zeiss ultra high-resolution LSCM to create “neat black” areas surrounding the cells. The cytoskeletal elements were examined in a blinded fashion for their overall morphology, orientation, and disruption as we have described (5, 6, 8, 12, 13). Two hundred cells per slide were examined in four different fields by LSCM, and the percentage of cells displaying normal microtubules was determined. Slides were decoded only after examination was complete.

Microtubule (tubulin) fractionation and quantitative immunoblotting of tubulin assembly and disassembly. Polymerized (S2) and monomeric (S1) fractions of tubulin were isolated after a series of centrifugation and extraction steps as we have described (5, 6, 8, 13). Fractionated S1 and S2 samples were then flash frozen in liquid N2 and stored at −70°C until immunoblotting. For immunoblotting, samples (5 µg protein per lane) were placed in a standard SDS sample buffer, boiled for 5 min, and then subjected to PAGE on 7.5% gels. Procedures for Western blotting were performed as previously described (5, 6, 8, 13). To quantify the relative levels of tubulin, the optical density of the bands corresponding to immunoradiolabeled tubulin were measured with a laser densitometer.

Determination of barrier permeability by fluorometry. Barrier integrity was determined by a widely used and validated technique that measures the apical-to-basolateral paracellular flux of fluorescent markers, such as fluorescein sulfonic acid (FSA, 200 µg/ml; 0.478 kDa), as we (5–8, 9–11, 15) and others (33, 35, 52, 56) have described. In select experiments, higher molecular mass fluorescein dextran (FD) probes such as the 4 kDa FD and 70 kDa FD (1 mg/ml) were also utilized. After treatments, fluorescent signals from samples were quantitated by a fluorescence multiplate reader (FL 600, BIO-TEK Instruments).

Statistical analysis. Data are presented as means ± SE. All experiments were carried out with a sample size of at least four to six observations per group. Statistical analysis comparing treatment groups was performed using ANOVA followed by Dunnett’s multiple range test (27). Correlation analyses were done using the Pearson test for parametric analysis or, when applicable, the Spearman test for nonparametric analysis. P values < 0.05 were deemed statistically significant.

RESULTS

Stable overexpression of PKC-ζ isoform. Intestinal cells were cotransfected with complementary DNA (cDNA) encoding both G-418 resistance (for selection) and PKC-ζ. Western immunoblotting analysis of cell lysates of these transfected cells from confluent monolayers demonstrates (Fig. 1A) the overexpression of the PKC-ζ isoform (3 µg of DNA plasmid shown). The PKC-ζ isoform isolated from transfected cells ran at the expected molecular mass of 72 kDa as confirmed by a known positive control for PKC-ζ. Identity of the PKC-ζ band was further ascertained by using the PKC-ζ blocking peptide in combination with the anti-PKC-ζ antibody that prevented the appearance of the corresponding major band in the Western blots. Additionally, in the absence of the primary antibody to PKC-ζ, no corresponding band for PKC-ζ was observed. Immunoblotting assessment of PKC-ζ protein levels (Fig. 1B) showed that total levels of this overexpressed isoenzyme were increased by ∼2.9-fold compared with wild-type cells. Optical densities (means ± SE) for these PKC-ζ overexpression studies were 12,210 ± 118 vs. wild type 4,175 ± 79. Preliminary studies confirmed that overexpression of PKC-ζ did not injure intestinal cells as indicated by a lack of change in viability assessed by ethidium homodimer-1 probe (5, 6).

Protective effects of the overexpressed PKC-ζ isoform against oxidant-induced injury. Multiple clones of intestinal Caco-2 cells (Table 1) or HT-29 cells (Table 2) transfected with 1, 2, 3, 4, or 5 µg of PKC-ζ sense cDNA showed a dose-dependent protection of barrier integrity in monolayers against oxidant-induced injury as assessed by FSA clearance. In Caco-2 monolayers, the clone transfected with 3 µg of PKC-ζ sense cDNA showed a 3.6-fold compared with wild-type cells. Optical densities (means ± SE) for these PKC-ζ overexpression studies were 12,210 ± 118 vs. wild type 4,175 ± 79. Preliminary studies confirmed that overexpression of PKC-ζ did not injure intestinal cells as indicated by a lack of change in viability assessed by ethidium homodimer-1 probe (5, 6).

Overexpression of PKC-ζ by itself did not deleteriously affect monolayer barrier function (Fig. 2A). Overexpression of PKC-ζ by itself did afford protection of barrier integrity (Fig. 2, A and B and Table 1) and the microtubule cytoskeleton (Fig. 3A and Table 3) against oxidant injury. This protection did not require the presence of EGF in the cell media. Only in cells stably overexpressing PKC-ζ was monolayer barrier integrity protected against oxidant injury
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Table 1. Effects of transfection of varying amounts of PKC-ζ sense or antisense DNA on Caco-2 monolayer barrier integrity

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSA Clearance (nl/h/cm²)</th>
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<tr>
<td>Vehicle</td>
<td>17 ± 5</td>
</tr>
<tr>
<td>H₂O₂, WT</td>
<td>824 ± 22†‡</td>
</tr>
<tr>
<td>H₂O₂, 1 μg sense ζ-DNA</td>
<td>773 ± 18‡†</td>
</tr>
<tr>
<td>H₂O₂, 2 μg sense ζ-DNA</td>
<td>526 ± 15†††‡‡</td>
</tr>
<tr>
<td>H₂O₂, 3 μg sense ζ-DNA</td>
<td>312 ± 39†</td>
</tr>
<tr>
<td>H₂O₂, 5 μg sense ζ-DNA</td>
<td>321 ± 31†</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, WT</td>
<td>46 ± 7††</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 1 μg antisense ζ-DNA</td>
<td>117 ± 14†††</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 2 μg antisense ζ-DNA</td>
<td>228 ± 14†††</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 3 μg antisense ζ-DNA</td>
<td>402 ± 33†</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 5 μg antisense ζ-DNA</td>
<td>397 ± 17†</td>
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Values are means ± SE after treatments; n = 4–6 observations per group. Intestinal Caco-2 cells stably transfected with varying amounts of protein kinase C (PKC-ζ) sense DNA (1, 2, 3, or 5 μg) were exposed to oxidant (H₂O₂, 0.5 mM) for 30 min. In separate studies, cells transfected with varying amounts of PKC-ζ antisense (1, 2, 3, or 5 μg) were treated with epidermal growth factor (EGF; 10 ng/ml) before exposure to oxidant. Select treatments from wild type (WT); untransfected cell monolayers are also shown. Monolayer barrier integrity was assessed by fluorescein sulfonic acid (FSA) clearance. *P < 0.05 compared with vehicle; †P < 0.05 compared with H₂O₂ in WT cells; ‡P < 0.05 compared with corresponding cells transfected with 3 μg of sense PKC-ζ DNA and exposed to H₂O₂ or cells transfected with 3 μg of PKC-ζ anti-sense DNA and treated with EGF and then H₂O₂.

Table 2. Effects of transfection of varying amounts of PKC-ζ sense or antisense plasmids on monolayer barrier integrity of another intestinal cell line, HT-29

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FSA Clearance (nl/h/cm²)</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>6 ± 10</td>
</tr>
<tr>
<td>H₂O₂, WT</td>
<td>798 ± 31*</td>
</tr>
<tr>
<td>H₂O₂, 1 μg sense ζ-DNA</td>
<td>780 ± 16†</td>
</tr>
<tr>
<td>H₂O₂, 2 μg sense ζ-DNA</td>
<td>543 ± 23††</td>
</tr>
<tr>
<td>H₂O₂, 3 μg sense ζ-DNA</td>
<td>287 ± 19†</td>
</tr>
<tr>
<td>H₂O₂, 4 μg sense ζ-DNA</td>
<td>276 ± 35†</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, WT</td>
<td>52 ± 9††</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 1 μg antisense ζ-DNA</td>
<td>97 ± 21†††</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 2 μg antisense ζ-DNA</td>
<td>189 ± 17†††</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 3 μg antisense ζ-DNA</td>
<td>365 ± 26†</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H₂O₂, 4 μg antisense ζ-DNA</td>
<td>342 ± 31†</td>
</tr>
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Values are means ± SE following treatments; n = 4–5 observations per group. Human colonic HT-29 cells transfected with varying amounts of PKC-ζ sense plasmid (1, 2, 3, or 4 μg) were incubated (30 min with H₂O₂ (0.5 mM). In other studies, cells transfected with varying amounts of PKC-ζ sense antisense (1, 2, 3, or 4 μg) were pretreated with EGF (10 ng/ml) and then exposed to H₂O₂. WT cell monolayers are also shown. Monolayer barrier function was determined by the clearance of FSA. *P < 0.05 vs. vehicle; †P < 0.05 vs. H₂O₂ in WT cells; ‡P < 0.05 vs. corresponding cells transfected with 3 μg of sense PKC-ζ and exposed to H₂O₂ or cells transfected with 3 μg of PKC-ζ antisense and treated with EGF and then H₂O₂.

as determined by decreased FSA clearance (62% lower clearance; Fig. 2A). Incubation with a concentration of EGF (1 ng/ml) that did not by itself afford significant protection, potentiated monolayer barrier protection in transfected cells overexpressing PKC-ζ. Wild-type cells (those not overexpressing PKC-ζ) on the other hand, had their barrier integrity protected against oxidant injury only by a high dose of EGF (10 ng/ml; Fig. 2A). As expected, transfection of only the SP-72 vector did not confer protection against exposure to oxidant (FSA clearance = 17 ± 5 nl/h/cm² for vector-transfected cells exposed to vehicle; 825 ± 23 nl/h/cm² for vector-transfected cells exposed to H₂O₂ alone vs. 312 ± 31 nl/h/cm² for PKC-ζ sense-transfected cells incubated in H₂O₂). This did not appear to be caused by changes in the ability of oxidants to cause damage because vector-transfected cells and wild-type cells responded in a similar fashion to H₂O₂, exhibiting comparable and significant barrier hyperpermeability.

Comparison of the protective effects of PKC-ζ overexpression on barrier integrity using several different molecular mass FD probes, including both 4 and 70 kDa FD probes, were consistent with the FSA clearance measures.
measurements (Fig. 2B). As expected, there was a molecular mass-dependent decrease in the permeation of these probes in order of increasing size (0.478 kDa FSA > 4 kDa FD > 70 kDa FD).

PKC-ζ overexpression by itself conferred protection to the microtubule cytoskeleton as demonstrated by the high percentage of intestinal cells displaying normal microtubules (Fig. 3A and Table 3). As for protection of barrier integrity in wild-type cells, microtubules were protected against oxidant exposure only by high doses (10 ng/ml) of EGF (Fig. 3A). Furthermore, transfection of SP-72 vector alone did not confer protection to the microtubules (%normal microtubules = 95 ± 5% for vector-transfected cells exposed to vehicle; 39 ± 3% for vector-transfected cells exposed to H2O2; 81 ± 4% for PKC-ζ sense-transfected cells exposed to H2O2). This did not appear to be due to changes in the ability of oxidants to cause damage because both vector-transfected cells and untransfected cells responded in a comparable manner to oxidant.

High-resolution LCSM of immunofluorescently stained microtubules also shows (Fig. 3B) that Caco-2 cells overexpressing PKC-ζ exhibit a normal cytoskeleton in cell monolayers exposed to oxidant (Fig. 3B, c). This protection is shown by the intracellular appearance of a normal stellate and radial cytoarchitecture of the microtubule cytoskeleton originating from the perinuclear region (i.e., microtubule organizer center) (Fig. 3B, c). Similarly, untreated wild-type cells (Fig. 3B, a) or untreated PKC-ζ-overexpressing cells (Fig. 3B, d) also showed an intact microtubule network dispersing throughout the cytosol. In the absence of PKC-ζ overexpression, wild-type cells exposed to H2O2 showed fragmentation, disruption, and collapse of the microtubules (Fig. 3B, b).

Fig. 2. A: overexpression of PKC-ζ protects the barrier integrity of Caco-2 monolayers against oxidant injury. Intestinal monolayers stably overexpressing PKC-ζ were incubated with epidermal growth factor (EGF) before exposure to oxidant H2O2 (0.5 mM). Transfected cells overexpressing PKC-ζ (Z) maintain their barrier integrity against oxidant-induced injury. WT monolayers, in contrast, were protected only by a high dose of EGF (10 ng/ml), whereas a low dose of EGF (1 ng/ml) did not protect WT cells. Also, note potentiation-induced protection of barrier integrity in PKC-ζ-overexpressing cells exposed to EGF. Both PKC-ζ-overexpressing cells and WT cells responded in the same fashion to vehicle treatment. Barrier integrity was expressed as flux of the fluorescent probe fluorescein sulfonic acid (FSA) from the apical to basolateral compartment of cell culture transwell inserts divided by the concentration of probe in the apical chamber. When normalized for the surface area of the monolayer, this expression has units of clearance. *P < 0.05 vs. vehicle; +P < 0.05 vs. H2O2 in WT; &P < 0.05 vs. PKC-ζ-overexpressing Z cells exposed to H2O2 or EGF before H2O2 in WT cells; #P < 0.05 vs. EGF (10 ng/ml) before H2O2 in WT cells (n = 6 cells per group). B: comparison of the protective effects of PKC-ζ overexpression on monolayer barrier integrity of Caco-2 cells exposed to oxidant H2O2 (0.5 mM) as determined by several fluorescein-conjugated probes of different molecular mass: 70 kDa fluorescein dextran (FD), 4 kDa FD, and 0.478 kDa FSA. Note the size (molecular mass)-dependent permeation of these probes in both WT and transfected Caco-2 cells. There is an inverse relationship between the probe size and clearance in order of increasing size (0.478 kDa FSA > 4 kDa FD > 70 kDa FD). *P < 0.05 vs. corresponding vehicle; +P < 0.05 vs. corresponding H2O2 in WT (n = 6 cells per group).
Fig. 3. A: percent of Caco-2 cells displaying normal microtubule cytoskeleton in PKC-ζ-overexpressing cells. Treatments and conditions were as explained in Fig. 2A. Cell monolayers grown on coverslips were processed for immunofluorescent staining with a primary monoclonal anti-β-tubulin antibody, and subsequently the microtubule elements were examined in a blinded fashion for their overall morphology. Microtubules are protected against oxidant injury in PKC-ζ-overexpressing cells. A high dose of EGF (10 ng/ml), which protects microtubules in WT cells, is also shown. PKC-ζ overexpression by itself is protective. Note the potentiation of protection in PKC-ζ-overexpressing cells exposed to EGF. PKC-ζ-overexpressing cells and WT cells responded comparably with vehicle treatment. *P < 0.05 vs. vehicle; †P < 0.05 vs. H₂O₂ in WT; ‡P < 0.05 vs. PKC-ζ Z cells exposed to H₂O₂ or EGF before H₂O₂ in WT cells; §P < 0.05 vs. EGF (10 ng/ml) before H₂O₂ in WT cells (n = 6 cells per group). B: intracellular distribution of the microtubules as imaged by ultra high-resolution laser confocal microscopy (LSCM) in intestinal cells from monolayers. Monolayers of WT Caco-2 cells were incubated with vehicle (isotonic saline) (a) or 0.5 mM H₂O₂ (b). PKC-ζ-overexpressing monolayers were exposed to 0.5 mM H₂O₂ (c) or vehicle (d). Microtubules in controls (a and d) appear as normal microfilamentous structures that disperse throughout the cytosol. In cells exposed to H₂O₂ (b), the microtubules show a clear collapse, disorganization, and disruption of their architecture. In contrast, in cells overexpressing PKC-ζ and incubated with oxidant, intact microtubule cytoarchitecture are highly preserved and protected, resembling the controls (c). Cells from desired areas of monolayers were processed using the image processing software on a Zeiss LSCM so as to create “neat black” areas surrounding the cells. Shown is a representative photomicrograph. Bar = 25 μm; n = 6 cells per group.
To determine effects of PKC-ζ overexpression on the dynamic alterations in the polymerization and depolymerization states of the microtubule cytoskeleton, we performed quantitative Western immunoblotting of tubulin, the structural protein of microtubules. To this end, the polymerized tubulin fraction (S2, an index of microtubule stability) and the monomeric tubulin (S1, an index of microtubule disruption) were isolated.

Quantitative immunoblotting of these fractions (Fig. 4A and Table 3) corroborated the microtubule studies noted above. Only the PKC-ζ-transfected oxidant-treated cells showed a stable tubulin assembly and intact microtubules (comparable with controls) as indicated by an increase in the polymerized S2 tubulin and a reduction in the monomeric S1 tubulin. In wild-type cells, in contrast, H2O2 decreased polymerized S2 tubulin and increased monomeric S1 tubulin, indicating disassembly of the microtubules. In these wild-type cells, only pretreatment with the higher doses (10 ng/ml) of EGF resulted in stable tubulin assembly. Transfection of vector alone, similar to its lack of protective effects on microtubules and barrier function, was ineffective in protecting tubulin assembly (*e.g., %tubulin assembly = 65 ± 0.5% for vector-transfected Caco-2 cells exposed to vehicle; 46 ± 0.8% for vector-transfected cells exposed to H2O2; 61 ± 0.6% for PKC-ζ sense-transfected cells exposed to H2O2).

Table 3. Transfection of PKC-ζ sense plasmid protects the microtubule cytoskeleton and tubulin assembly while antisense inhibits protection in intestinal HT-29 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>%Normal Tubulin</th>
<th>%Polymerized Tubulin</th>
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<tbody>
<tr>
<td>Vehicle</td>
<td>98 ± 2</td>
<td>67 ± 0.35</td>
</tr>
<tr>
<td>H2O2, WT</td>
<td>45 ± 4*</td>
<td>48 ± 1.20‡</td>
</tr>
<tr>
<td>H2O2, 3 μg sense ζ-DNA</td>
<td>82 ± 2†‡</td>
<td>62.5 ± 0.80†</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H2O2, WT</td>
<td>90 ± 3†</td>
<td>65 ± 0.50†</td>
</tr>
<tr>
<td>EGF, 10 ng/ml + H2O2,</td>
<td>63 ± 6*‡‡</td>
<td>54.5 ± 0.90†*‡‡</td>
</tr>
</tbody>
</table>

Values are means ± SE following treatments; n = 4–5 observations per group. Human colonic HT-29 clone transfected with sense plasmid (3 μg) for PKC-ζ isoform was incubated (30 min) with H2O2 (0.5 mM). Another clone transfected with antisense plasmid (3 μg) for PKC-ζ was preincubated with EGF (10 ng/ml) and then H2O2. WT cells are also shown. %Normal microtubule cytoskeleton and %polymerized tubulin were subsequently assessed. *P < 0.05 vs. vehicle; †P < 0.05 vs. H2O2 in WT cells; ‡P < 0.05 vs. EGF + H2O2 in WT cells.

Fig. 4. A: immunoblotting analysis of the polymerized tubulin (S2, index of microtubule assembly) and the monomeric tubulin (S1, index of microtubule disassembly) in Caco-2 cell monolayers overexpressing atypical PKC-ζ. Tubulin fractions were extracted from monolayers and subjected to SDS-PAGE fractionation and immunoblotted using monoclonal anti-β-tubulin antibody followed by horseradish peroxidase conjugated-secondary antibody and subsequently autoradiographed. To quantify the relative levels of tubulin bands, the optical density of the bands corresponding to immunoradiolabeled tubulin were measured with a laser densitometer. Results for %tubulin assembly for WT cell extracts are also shown. Conditions were as described in Figs. 2A and 3A. Percent polymerization of tubulin is [(S2)/(S2 + S1)], where S2 + S1 is the total cellular tubulin pool. †P < 0.05 vs. vehicle; ‡P < 0.05 vs. H2O2 in wild type; &P < 0.05 vs. Z cells exposed to H2O2 or EGF before H2O2 in WT cells; #P < 0.05 vs. EGF (10 ng/ml) before H2O2 in WT cells (n = 6 per group). B and C: photomicrographs representing typical Western immunobLOTS of the polymerized tubulin/S2 fractions (B) and monomeric tubulin/S1 fractions (C) from PKC-ζ-transfected and WT intestinal Caco-2 monolayers. Tubulin fractions extracted from intestinal cells were processed for Western immunoblotting as described in Fig. 4A. Immunobotted tubulin on nitrocellulose membranes was visualized by enhanced chemiluminescence (ECL) and autoradiography. The tubulin bands from left to right correspond to: α, WT cells exposed vehicle; β, PKC-ζ-overexpressing cells exposed to vehicle; γ, WT cells exposed to 0.5 mM H2O2; δ, PKC-ζ-overexpressing cells exposed to 0.5 mM H2O2 and 0.5 mM H2O2; ε, WT cells treated with EGF (1 ng/ml) + 0.5 mM H2O2; χ, PKC-ζ-overexpressing cells treated with EGF (1 ng/ml) + 0.5 mM H2O2; γ, WT cells treated with EGF (10 ng/ml) + 0.5 mM H2O2; δ, PKC-ζ-overexpressing cells treated with EGF (10 ng/ml) + 0.5 mM H2O2; ε, tubulin standard (50 kDa). PKC-ζ overexpression in transfected cells by itself maintains normal dynamics of tubulin polymerization against oxidant-induced disassembly. This is shown by a tubulin band density comparable to that of the control (vehicle) levels. In WT cells, only a high dose of EGF, but not a low dose, enhanced tubulin assembly to levels similar to that of transfected or control cells. Shown is a representative blot from each tubulin fraction (n = 6 per group).
Figure 4, B and C shows representative Western blots of the actual alterations in tubulin pools, again demonstrating that PKC-ζ overexpression enhances the S2 tubulin (Fig. 4B) band density to a level comparable with that of controls and reduces S1 tubulin band density (Fig. 4C), indicating increased polymerization of tubulin and stabilization of microtubules. Oxidant exposure decreased the S2 tubulin band density well below the control levels. These findings parallel the protective effects of PKC-ζ overexpression on microtubule integrity and on barrier permeability.

Intracellular distribution and constitutive activation of the overexpressed PKC-ζ in transfected intestinal monolayers. Western immunoblotting assessment of the cytosolic, membrane, and cytoskeletal-associated fractions from transfected cells overexpressing PKC-ζ showed that the ζ-isofrom (72 kDa) is found mostly in the membrane and cytoskeletal fractions of these transfected cells with only a small distribution to the cytosolic fractions (Fig. 5A, Caco-2 shown). In wild-type cells (Fig. 5B), in contrast, we found a mostly cytosolic distribution of PKC-ζ with smaller pools in the membrane and cytoskeletal (particulate) fractions, indicating inactivity. Figure 6 shows a graphic depiction of the intracellular distribution of the overexpressed PKC-ζ in various Caco-2 cell monolayer fractions as a fraction of total distribution (expressed in arbitrary units).

Finding PKC-ζ in particulate pools indicates that the overexpressed PKC-ζ isoform is “constitutively active” because achieving this distribution by PKC-ζ did not require EGF or OAG (a PKC activator). Pretreatment of transfected cells with EGF, however, further increased the fraction of PKC-ζ isoform into the membrane and cytoskeletal fractions, reaching near-total activation of PKC-ζ. Wild-type cells exposed to vehicle or oxidant show a mostly cytosolic distribution of PKC-ζ. In these wild-type cells, we noted rapid translocation of native PKC-ζ into particulate (membrane +
cytoskeletal) fractions of cells only after exposure to high doses of EGF, which confirms our recent and preliminary findings (8).

Using data across all experimental conditions, there was a significant \( P < 0.05 \) inverse correlation \( r = -0.90 \) between PKC-\( \zeta \) levels (optical density from the particulate fraction) and probe clearance, suggesting that constitutive activation of the \( \zeta \)-isoform may be important in protection of barrier permeability. Similarly, we found other robust (positive) correlations when either microtubule integrity or tubulin assembly were correlated with the PKC-\( \zeta \) levels \( r = 0.89, 0.91 \), respectively, \( P < 0.05 \) for each).

**Stable antisense inhibition of PKC-\( \zeta \) to underexpress the \( \zeta \)-isoform and its prevention of EGF-induced protective effects.** The above findings indicate that PKC-\( \zeta \) might, by itself, play a key role in cellular protection. To show that PKC-\( \zeta \) specifically contributes to EGF-mediated protection, we utilized an antisense approach to stably decrease the steady-state levels of PKC-\( \zeta \) protein. Figure 7A shows an immunoblot of cell lysates of wild-type Caco-2 cells transfected with PKC-\( \zeta \) antisense cDNA (3 \( \mu \)g) and plasmid encoding G-418 resistance. These data show a substantial reduction \((-95%)\) in the levels of PKC-\( \zeta \) protein in these antisense-transfected cells.

PKC-\( \zeta \) underexpression by itself did not deleteriously affect Caco-2 monolayer barrier integrity (FSA clearance; Fig. 7B and Table 1). Antisense inhibition of expression of the PKC-\( \zeta \) protein did, however, substantially and significantly attenuate the protection afforded by 10 ng/ml EGF (reduction to \(-48 \pm 8%\) protection). Thus \(-50\%\) of EGF-afforded protection appears to be PKC-\( \zeta \) dependent and \(-50\%\) to be PKC-\( \zeta \) independent (Fig. 7B and Table 1). We observed similar effects by antisense transfection in another intestinal cell line, HT-29 (Table 2).

Tables 1 and 2 also show the dose-dependent effects of varying amounts (1, 2, 3, 4, or 5 \( \mu \)g) of PKC-\( \zeta \) antisense plasmid on inhibition of EGF-induced protection in intestinal cells. The clone transfected with 3 \( \mu \)g plasmid for PKC-\( \zeta \) antisense in both Caco-2 cells and HT-29 cells provided maximum inhibition of EGF-induced protection, and it was thus used for subsequent antisense studies.

In parallel, analysis of the percentage of antisense-transfected cells with a normal microtubule cytoskeleton demonstrates (Figs. 8, A and B, and Table 3) that antisense inhibition of PKC-\( \zeta \) expression prevented protection of microtubules by a high dose of EGF. PKC-\( \zeta \) isoform underexpression by itself did not damage the microtubules.

Quantitative Western immunoblotting of tubulin from the antisense-transfected cells further demonstrates (Fig. 9A and Table 3) that in the absence of the PKC-\( \zeta \) isoform EGF does not elicit any increases in the stable S2 tubulin fraction (nor any decreases in monomeric S1 tubulin), again indicating prevention of microtubule assembly and integrity.

Two representative Western blot photomicrographs further confirm (Fig. 9, B and C) that with the underexpression of PKC-\( \zeta \) isoform, EGF neither causes the enhancement of the S2 tubulin (Fig. 9B) band density (to the level comparable to that of controls) nor leads to the reduction in S1 tubulin (Fig. 9C).
DISCUSSION

We have demonstrated that the \( \zeta \) -isoform of PKC plays an important role in EGF-mediated protection against oxidant damage to the microtubule cytoskeleton and to cell monolayer integrity. This isoform of PKC also appears to be a critical endogenous stabilizer of both cytoskeletal and barrier function. Several lines of evidence in the current study support the aforementioned findings.

First, overexpression of PKC-\( \zeta \) induces an EGF-like protection against oxidant-induced disruption of bar-

Fig. 8. A: stable antisense inhibition of PKC-\( \zeta \) protein expression inhibits the protective effects of EGF on the microtubule cytoskeleton. The percentage of Caco-2 cells in monolayers displaying normal microtubule cytoskeletons was assessed in antisense-transfected cells treated with EGF (10 ng/ml) before oxidant (conditions as described in Fig. 7B). \( * P < 0.05 \) vs. vehicle; \( + P < 0.05 \) vs. \( \text{H}_2\text{O}_2 \); \( \& P < 0.05 \) vs. EGF + \( \text{H}_2\text{O}_2 \) in WT cells (\( n = 6 \) cells per group). B: ultra high-resolution laser confocal microscopy of the microtubule cytoarchitecture in PKC-\( \zeta \) antisense-transfected and WT intestinal cells from monolayers. Monolayers of WT Caco-2 cells were preincubated with EGF (10 ng/ml) and then 0.5 mM \( \text{H}_2\text{O}_2 \) (a). PKC-\( \zeta \) -underexpressing monolayers were pretreated with EGF (10 ng/ml) plus \( \text{H}_2\text{O}_2 \) (b) or vehicle (c). Microtubules in EGF-pretreated WT cells (a) appear protected as shown by their intact architecture. In contrast, in antisense-transfected cells the same dose of EGF is not protective (b). Here, the microtubules show collapse and fragmentation. Underexpression of PKC-\( \zeta \) by itself does not deleteriously effect the microtubule cytoskeleton (c). Shown is a representative photomicrograph. Bar = 25 \( \mu \text{m} \); \( n = 6 \) cells per group.
rrier integrity. This protection appears to require overexpression and constitutive activation of PKC-ζ. In particular, protection is dependent on constitutive activation through the distribution of PKC-ζ into the particulate (cytoskeletal + membrane) fractions. Second, overexpression of PKC-ζ induces stabilization of the microtubule cytoskeleton, a protective phenomenon we have shown to be key in the maintenance of cell monolayer integrity. Overexpression of PKC-ζ decreases the unstable monomeric (S1) tubulin, increases the stability of polymerized (S2) tubulin, and increases the percentage of Caco-2 cells displaying normal microtubules. Third, a low, nonprotective concentration of EGF potentiates all measures of PKC-ζ-induced protection. Fourth, antisense inhibition of the expression of PKC-ζ reduces EGF protection of barrier integrity by ~48 ± 8%, the remaining 52% of EGF protection apparently being PKC-ζ independent. In these antisense-transfected clones, which expressed ζ isoform at ~5% of wild-type levels, EGF protection of S2 tubulin assembly and microtubules was also significantly prevented. Fifth, increases in expression of PKC-ζ quantitatively correlate with increases in outcomes indicating protection (barrier integrity, tubulin polymerization, microtubule assembly, and integrity of microtubule cytoarchitecture).

Our findings are consistent with our previous reports that activation of PKC in general is required for EGF protection (8) and that specific isoforms mediate that protection (11). For example, PKC-β1 isozyme mediates a substantial portion (60 ± 8%) of EGF protection (11). On the basis of percent mediation of protection, it is reasonable to speculate that activation of both β1 and ζ isoforms of PKC can account for 100% of EGF-induced protection.

Although PKC-β1 and PKC-ζ share in common the ability to protect, there appear to be differences in their mechanisms of action. Protection by PKC-ζ, as shown herein, does not require the presence of pharmacological activators of PKC (e.g., OAG or EGF), whereas protection by PKC-β1 does require them (8, 11). This difference is fully consistent with the fact that PKC-ζ is an “atypical” isoform of PKC, whereas PKC-β1 is a “conventional” isoform. Indeed, our findings on the atypical PKC-ζ are consistent with reports in non-GI models in which the ζ isoform activation was shown to be independent of PKC activators (e.g., OAG or 12-O-tetradecanoylphorbol 13-acetate) (18, 25, 50). For example, the activation of PKC-ζ is not dependent on treatment with phorbol esters or DAG (OAG) (25). Similarly, atypical PKC isoforms λ and τ do not respond to phorbol esters or OAG (21). In contrast, DAG has been shown to induce activation of classic isoforms of PKC, such as β1, in non-GI cellular models (e.g., fibroblasts) (25) as well as in GI cells (e.g., Caco-2 cells) as we recently reported (11).

Our findings regarding the subcellular distribution of PKC isoforms are consistent with known biochemical properties of PKC isoforms. All PKCs consist of NH2-terminal regulatory domains and COOH-terminal catalytic domains (separated by a flexible hinge

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Fig. 9. A: antisense inhibition of PKC-ζ protein inhibits the enhancement of tubulin assembly by EGF as determined by quantitative immunoblotting analysis of tubulin pools from Caco-2 cells. Both the polymerized tubulin (S2, index of assembly) and monomeric tubulin (S1, index of disruption) were assessed. Conditions were similar to those in Fig. 8A. Percent polymerization of tubulin = [(S2)/(S2 + S1)]. *P < 0.05 vs. vehicle; +P < 0.05 vs. H2O2; &P < 0.05 vs. EGF + H2O2 in WT cells (n = 6 cells per group). Representative Western blot photomicrographs of the polymerized S2 tubulin (triton-insoluble) extracts (B) and monomeric S1 tubulin extracts (C) following similar treatment regimens as in Fig. 8A are shown. Tubulin fractions from Caco-2 cells were analyzed by SDS-PAGE and Western immunoblots and subsequently processed for X-ray film exposure. The lanes from right to left are: a, WT cells exposed to vehicle; b, PKC-ζ-underexpressing cells exposed to vehicle; c, WT cells exposed to 0.5 mM H2O2; d, PKC-ζ-underexpressing cells exposed to 0.5 mM H2O2; e, WT cells treated with EGF (10 ng/ml) + 0.5 mM H2O2; f, PKC-ζ-underexpressing cells treated with EGF (10 ng/ml) + 0.5 mM H2O2; and g, tubulin standard (50 kDa). Shown is a representative immunoblot from each tubulin extract (n = 6 cells per group).

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region) (26). In resting cells, PKC is mainly found in an inactive conformation. In this inactive phase, PKC is mainly distributed in the soluble (cytosolic) fraction and only loosely bound to membrane components. Regulatory domains of PKC isoforms vary from one subfamily to the next as well as among individual isoforms within a given subfamily (21, 26, 47). For example, OAG (or DAG) binding sites are present in the regulatory “zinc finger” domain of PKC-β1 but are absent from the regulatory domain of PKC-ζ. Not surprisingly, PKC-ζ has severalfold lower affinity for OAG than has PKC-β1 at the zinc finger domains (26). Additionally, in many wild-type cells, PKC-β1 and PKC-ζ appear to be found in different subcellular fractions (11, 21). Consistent with these known facts, our previous and present studies collectively demonstrate a novel concept that increased levels of these PKC isoforms in the particulate fractions (i.e., activation) either by pharmacological manipulation (OAG or EGF for PKC-β1) or by transfection (constitutive activation for PKC-ζ) lead to enhanced cellular protection.

Although most cells express more than one type of PKC isoform, differences among isozymes with respect to activation conditions and subcellular locations suggest that individual PKC isoforms have distinct activation mechanisms as well as mediate distinct biological processes (1, 4, 16, 17, 32, 41, 44, 46, 49–51, 60). In resting cells (in the absence of lipid cofactors), most PKCs assume an inactive structural conformation. This is maintained by an intramolecular interaction between an autoinhibitory sequence (i.e., the pseudosubstrate on the NH2 terminus) in the regulatory domain and the substrate-binding region of the catalytic domain (26). Moreover, accumulated evidence suggests that modifications of the regulatory domain of a PKC isoform can lead to the activation of that isoform (21, 26). For example, binding of PKC-ζ isoform to phospholipids, especially anionic phosphotidylserine, in membranes (i.e., translocation to membranes) is thought to be necessary to cause conformational changes to its regulatory domain, before activation. Specifically, structural studies (26) demonstrate that in the presence of inducers (e.g., overexpression by transfection), this regulatory domain (especially the zinc finger portion) forms an automatic hairpin-like hydrophobic structure that mediates PKC interaction with the membrane lipids and subsequent conformational changes within the regulatory domain, leading to autoactivation. Thus overexpression of PKC-ζ may promote a conformational change that releases the inherent autoinhibition present and triggers kinase catalytic activity. For DAG-dependent PKC isoforms such as PKC-β1 (11, 21), on the other hand, the inhibitory function of the regulatory domain can be overcome by pharmacological agents that mimic DAG (e.g., OAG or 12-O-tetradecanoylphorbol 13-acetate), thus producing conformational changes within this key domain and, in turn, resulting in an activated form of the PKC isoform.

Our findings that activation of PKC in general (8) and PKC-β1 (11) and PKC-ζ in particular are involved in protection of intestinal cells are supported by two recent pharmacological studies (54, 55). For example, Terres et al. (54) using intestinal T-84 cells showed that Helicobacter pylori-associated decreases in monolayer barrier resistance was inhibited in the presence of a PKC activator 12-O-tetradecanoylphorbol 13-acetate, thus suggesting a possible role for PKC in protection against bacterial-induced damage. Additionally, our findings on the β1-isoform of PKC (11) and ζ-isoform (current report) utilizing more specific and targeted molecular approaches further expand on these previous pharmacological reports and, we believe, now establish a novel biological function (protection) among the isoforms of PKC subfamilies. Furthermore, we have more recently identified activation of EGF receptor tyrosine kinase and then phospholipase C-γ1 as the upstream signal for EGF-induced, PKC-mediated protection of intestinal barrier and cytoskeletal integrity (10).

Our series of studies on PKC, to date, were designed to investigate possible beneficial effects of PKC isoform activation in the GI tract. Although our findings are consistent with other published pharmacological studies, several reports (3, 23, 47) have shown that activation of PKC in cellular models may lead to nonprotective effects and these may vary with different experimental conditions and cell types. For example, overexpression of PKC-δ leads to the disruption of pig kidney epithelial (LLC-PK1) cell monolayers (47). A recent pharmacological study also suggested that PKC-δ and PKC-ε appear to be involved in tumor necrosis factor-α-induced injury in intestinal (IEC-18) cells (59).

Our findings show that stable antisense inhibition of PKC-ζ prevents all measures of EGF-induced protection in our intestinal cell model. Whereas the mechanism for this inhibitory effect on EGF protection needs to be fully established, this attenuation is consistent with our data showing substantial downregulation of PKC-ζ expression by its antisense inhibition as well as by parallel inhibition of three separate EGF-related protective variables: barrier integrity, tubulin assembly, and microtubule stability. A question that remains to be answered is how antisense to PKC-ζ prevents EGF protection. We now suggest a mechanism by which the PKC-ζ downregulation prevents EGF-induced protection: protein phosphorylation by PKC-ζ by any of several well-known cellular mechanisms, such as cytoskeletal phosphorylation/dephosphorylation. This mechanism is consistent with our previous reports that EGF protection is mediated through stabilization of the assembly of the tubulin-based cytoskeleton (6, 8) and that EGF-induction causes PKC to phosphorylate tubulin and enhance tubulin assembly, which correlates significantly (r = 0.90 and 0.88, respectively; P < 0.05 for each) with EGF protection of microtubule stability and of barrier integrity (11). This proposed mechanism is further consistent with several reports in non-GI models that PKC activation phosphorylates and stabilizes cytoskeletal proteins (25, 28). Because cytoskeletal assembly and stability is critical in cellular protection, it follows that downregulation of PKC-ζ (by antisense) can prevent essential protein...
phosphorylation, thereby preventing EGF protection of cytoskeletal integrity. For example, we recently reported (11) that EGF or the PKC activator OAG led to an enhancement of serine phosphorylation of the tubulin (50 kDa) subunit protein of the microtubules. This increase was substantially attenuated by antisense lin (50 kDa) subunit protein of the microtubules. It is possible, therefore, that activated PKC-β1 or activated PKC-ζ phosphorylates the same or similar cytoskeletal or membrane targets. This proposed mechanism in our GI model is consistent with several previous studies in non-GI models. For instance, PKC has been shown to be involved in remodeling of the cytoskeletal filaments (2, 22, 25, 28, 40), although it has not been clearly established which PKC isoforms are essential in these processes. PKC can phosphorylate the cytoskeletal proteins talin and vinculin (25). Also, a specific substrate for PKC, myristoylated, alanine-rich PKC substrate protein (MARCKS), has been suggested to be an actin cytoskeletal reorganizer (28). In particular, MARCKS activity is abolished by PKC-induced phosphorylation. It is also possible that PKC isoforms can phosphorylate tubulin-associated capping proteins (e.g., microtubule associated proteins).

Evidence exists for other possible mechanisms for protection by PKC. Our previous reports showed that certain antioxidants (5, 7, 9) or agents that normalize intracellular calcium homeostasis (8) prevent oxidative damage in our model. Therefore, enhancement of either of these mechanisms could conceivably underlie PKC protective effects. Studies are underway in our laboratory to determine to what extent PKC protection is mediated by either of these mechanisms.

In summary, it appears that PKC-ζ is responsible for a substantial portion of normal protection of the GI mucosal epithelium and perhaps is key to preventing amplification and perpetuation of an uncontrolled, oxidant-induced, inflammatory cascade that can be ignited by free radicals and other oxidants present in the GI tract. By creating the first GI cells stably overexpressing “protective PKC isoforms,” our laboratory has discovered that these PKC isoforms possess critical functions in protecting cells against oxidative stress. This new knowledge may prove useful because increasing the activity of protective PKC isoforms through activation of endogenous PKC or using PKC mimetics may lead to novel therapeutic strategies for the treatment of a wide variety of oxidant-induced inflammatory disorders of the GI tract, including IBD.

Finally, our proposed mechanism of protection against oxidative stress has laid the groundwork for future “translational research” in humans and animals. We envision that these in vitro experiments will lead to highly focused studies that will test the clinical relevance of these potentially key biochemical pathways in IBD. For example, we (A. Keshavarzian, A. Banan, S. Kommandori, Y. Zhang, and J. Z. Fields, unpublished observations) have shown that a number of these oxidative reactions also occur in intestinal mucosa from patients with IBD. An important question that remains to be answered is whether modulation of PKC activity in vivo might also prevent oxidative damage.

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