Actin-based features negatively regulated by protein kinase C-ε

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Li, Yingxin, Jason M. Urban, Marilyn L. Cayer, Howard K. Plummer, III, and Carol A. Heckman. Actin-based features negatively regulated by protein kinase C-ε. Am J Physiol Cell Physiol 291: C1002–C1013, 2006; doi:10.1152/ajpcell.00079.2006.—Cells exposed to phorbol 12-myristate 13-acetate (PMA) undergo a choreographed sequence of morphological changes. Some of these, including stimulation of membrane ruffles and the later appearance of stress fibers, rely on remodeling of the actin cytoskeleton. Although this process is poorly understood, it is important, because the same features are affected during oncogenic transformation. PMA also activates protein kinase C (PKC). Enzyme activation is followed by degradation. Either affected during oncogenic transformation. PMA also activates protein kinase C (PKC). Enzyme activation is followed by degradation. Either process might affect the remodeling of actin. The present studies determined whether any PKC isozymes were subject to degradation in tracheal epithelial cells by quantifying the amount of each isozyme present after PMA exposure. PKC-ε was the only isozyme to show declining content correlated with increased stress fiber accumulation. Stress fibers increased between 5 and 10 h, whereas PKC-ε declined to 38% of its starting value (95% confidence interval, 10–68%). The relationship could be fit by the function \( F(x) = 0.683 \times \exp[-0.841(x - 0.387)] \), where \( F \) is the frequency of fiber-containing cells and \( x \) is PKC-ε content. Fiber accumulation was further investigated after knockdown of PKC-ε with RNA interference and antisense oligodeoxynucleotide. Knockdown enhanced stress fibers in cells not yet exposed to PMA as well as the final frequency of fiber-containing cells after PMA exposure. With knockdown at both transcriptional and proprotein levels, ~15% of the original content was predicted and achieved, as judged from real-time PCR and PKC-ε content measurements. The results suggest that PKC-ε negatively regulates stress fibers, either by directly turning over one of their components or by regulating an upstream step affecting fiber organization.

focal adhesion; focal contact; cell motility; shape analysis; tumor promotion

WORK FROM THIS LABORATORY has demonstrated that cancer cells have characteristic features that can be recognized by computer-assisted microscopy and sophisticated classification techniques. The same changes were induced by exposure to the phorbol ester PMA as had characterized cultured cells when they become oncogenically transformed. PMA-induced changes were transient, however, taking place over a time course of 10 h (44). The alterations in morphometric features were accompanied by changes that depended on assembly and reorganization of actin microfilaments. Included among these were augmentation of membrane ruffling, nonspecific endocytosis, and formation of p21–activated kinase (PAK)-dependent protrusions (19–21). Because the PKC family of enzymes constitute the major cellular receptors for PMA, some of these changes may involve PKC activation and PKC-mediated phosphorylation of an actin-binding protein. It is clear that PKC can phosphorylate many different proteins (37, 38), however. It has proved difficult to link phosphorylation of a specific substrate to a single physiological or morphological consequence. The current research demonstrates that such phosphorylation events can be linked to specific consequences for cell structure and function.

PKC is a family of serine, threonine kinases consisting of 12 distinct isozymes. PKCs are classified into groups, known as conventional isozymes (cPKCs), novel PKCs (nPKCs), and atypical PKCs (aPKCs). The cPKCs, \( \alpha, \beta I, \beta II, \) and \( \gamma \), are activated by \( \mathrm{Ca}^{2+}, \) diacylglycerol (DAG), and phosphatidylserine. The nPKCs, \( \delta, \epsilon, \eta, \) and \( \theta \), are activated by DAG and phosphatidylserine. Isozymes \( \lambda \) and \( \zeta \) (aPKCs) lack both \( \mathrm{Ca}^{2+} \) and DAG-binding sites and are activated by phosphatidylserine, phosphatidylinositol 3,4,5-trisphosphate, and unsaturated fatty acids. A fourth group, as yet unnamed, contains isozymes \( \mu \) and \( \nu \). These isozymes are activated by phosphatidylinositol 4,5-bisphosphate and DAG (45). PKC sequences include a number of variable and conserved (C) domains, of which the DAG-binding C1 domain is on the NH₂-terminal half of the molecule. Also on the NH₂-terminal half is a pseudosubstrate motif resembling the sequence found in phosphorylation sites of PKC substrates. This motif cannot be phosphorylated, however, and serves to bind the catalytic site. This binding is thought to fold PKC into a hairpin, which is unfolded when the enzyme is translocated to a membrane or protein scaffold, enabling substrate to get access to the active site.

DAG is produced in the cell by two different pathways. Ligand-receptor binding at the plasma membrane typically causes phospholipase C activation, which hydrolyzes phosphatidylinositol 4,5-bisphosphate to form 1,4,5-trisphosphate and DAG. This DAG disappears rapidly, but a second wave of DAG is formed by a phospholipase D-mediated pathway downstream of PKC-initiated signaling (reviewed in Ref. 33). Along with activation of its enzymatic activity, DAG and PMA cause PKC to unfold and translocate to specific sites in the membrane or cytoskeleton. Because the hinge region is sensitive to cleavage, the unfolded form of PKC is readily cleaved into two pieces (26). Although the catalytic portion of the enzyme is still active, its activity is rapidly downregulated (50). Studies on turnover of the intact enzyme have shown that the half-life of PKC was changed from >24 h to 48 min by PMA treatment (58). This is consistent with data on PKC-α showing

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that the catalytic fragment was degraded ~10 times faster than the intact molecule (52).

The availability of DAG or PMA affects the degradation of different isozymes in different cell types. Even within the same group of isozymes, which are assumed to be activated by the same second messenger, there are patterns of selective degradation. For example, in LLC-PK1 renal cells, PKC-δ is degraded, whereas PKC-ε is conserved (8). In the cPKC group, partial loss of PKC-α was observed in cultures where there was no loss of PKC-γ (34). In many cell types, the isozymes degraded include representatives of two or more groups. In GH3 pituitary cells, the combination of PKC-β and PKC-ε is lost, whereas other isozymes were unaffected (1). In SH-SY5Y neuroblastoma and embryonic 3T3-F442A cell lines, the content of PKC-ε was unaltered, but PKC-α alone or the combination of PKC-α and PKC-δ was degraded, respectively (34, 57). In mouse embryonic Swiss 3T3 and human Jurkat leukemia cells, the majority of nPKC content (PKC-δ and PKC-ε) is lost, but PKC-α is less likely to be degraded (35, 40). Similar diversity has been noted in the nature of isozymes translocating to the nucleus after activation (reviewed in Ref. 5).

Although such differences in susceptibility to degradation are poorly understood, PKC may translocate to different sites in different cell types. There is evidence of two different mechanisms of degradation (8, 27). In the case of either bradyostatin- or PMA-mediated activation, dephosphorylation of activated PKC-α and PKC-ε predisposes the protein to ubiquitination and targets it to the proteasome (27). Degradation did not appear to depend on lysosomal processing, vesicle trafficking, or the Ca^{2+}-activated cysteine proteinases called calpains in this case, but others have reported that certain isozymes, especially PKC-ε, were degraded by calpains (12).

Still other investigators found an alternative pathway of PKC-α downregulation through trafficking to an endosomal compartment (29, 48). Because an isozyme may be translocated to either a membrane or cytoskeletal site upon activation, in a cell type-specific way, the site to which the protein is translocated may be important for specifying the mechanism of degradation. Molecules in membranes may be targeted to the lysosome and those in the cytosol to the proteasome (49).

In these studies, we have exploited the coincidence between changes in actin architecture and the transient expression of transformed shape features in PMA-treated cells to learn more about functions related to transformation. The immediate effect of PMA exposure, in terms of actin organization, was activation of ruffling that was common to several different cell types (reviewed in Ref. 19). A study by Prekeris et al. (46) related PKC activation to ruffling stimulation and/or stress fiber dissolution. This stimulation was followed by downregulation of ruffling, and at the same time, stress fibers appeared. Such fibers were rarely observed in single tracheal epithelial cells from untreated cultures (21). Considering the pattern of selective activation and degradation of PKC isozymes following PMA exposure, we realized that activation of one or more isozymes could account for the transition between different types of actin architecture. We postulated that an isozyme of PK that normally turned over stress fibers was activated by PMA, and its subsequent degradation enabled their accumulation.

A content curve was measured for each of the seven PKC isozymes represented in rat tracheal cells by immunoprecipitation of the protein with a specific antibody and separating it with SDS-PAGE. PKC-ε showed rapid degradation between 5 and 10 h, which coincided with stress fiber accumulation in PMA-treated cells. The data were fit by an exponential curve relating the amount of PKC-ε in the cells to the frequency of stress fibers. The fit of fiber frequency by this inverse relationship was used to predict the amount of protein remaining after a knockdown protocol for PKC-ε. The results are consistent with the proposed role of PKC-ε in turning over stress fibers. This activity could be important, because the ubiquitous PKC-ε binding protein RACK1 also binds the normal homolog of the Src oncogene and modulates chemotactic migration (9). PKC-ε uniquely among PKC isoforms was included in a constitutive complex with Raf and inactive Ras (15). Such multiprotein complexes are formed and dissociated rapidly, making it difficult to study the mechanisms by which they regulate cell behavior and motility. Moreover, stress fibers are anchored in focal adhesion sites. An understanding of the mechanism of stress fiber dissolution by PKC-ε might provide insight into the mechanism of turnover of adhesive structures that is essential to cell cycling, motility, and chemotaxis.

**MATERIALS AND METHODS**

**Cell culture and sample preparation.** A line of tracheal epithelial cells, 1000 W, was derived from a heterotopic tracheal transplant in a male F344 rat exposed in vivo to 7,12-dimethylbenz(a)anthracene. The cells were cultured in Waymouth’s medium supplemented with 0.1 μg/ml hydrocortisone (Calbiochem, La Jolla, CA), 0.1 μg/ml insulin (Calbiochem), and 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA). Cells were maintained in the supplemented medium (WHIC) at 37°C in a humidified atmosphere of 5% CO₂ and 95% air and were subcultured at a density of 4 × 10⁵ cells per 60-mm dish as previously described (16). The line has been tested periodically for Mycoplasma and found to be negative.

For immunoprecipitation experiments, cells were plated at 10⁶ per 60-mm dish. After subculture, cells were incubated under normal growth conditions for at least 9 h to allow them to attach to dishes and were then transfected (see below). Later, cells were treated with 2 nM PMA (Sigma-Aldrich, St. Louis, MO) under the same conditions as described above and collected at various time intervals (21, 44). For studies on transcriptional inhibitors, amanitin and actinomycin D were used at concentrations of 7.5 and 0.2 μg/ml, respectively, for times of 10 or 15 h.

**Transfection with antisense oligodeoxynucleotides.** Phosphorothioate-modified oligodeoxynucleotides (ODNs) were purchased from Sigma Genosys (http://www.sigma-genosys.com). The antisense sequence used against PKA-α was 5'-GGTAAACGTCAGCATG-GTTCT-3' [6,446 daltons, bases 215–235, GenBank accession no. NM_343975 (UniGene RN.86669)]. An antisense ODN against PKC-β, 5'-CGCGCGGCGGTTGTCACTTGTGTC-3' [bases 87–104, accession no. NM_012713 (UniGene RN.91118)], was modified from a sequence previously reported to cause 40% knockdown of the human protein (28). Antisense ODN against PKC-ε, 5'-ATTGAACACTAC-GGTCCG-3' [bases 194–208, accession no. NM_017171 (UniGene RN.343966)], was the same sequence used by Cheng et al. (7), who reported knockdown of the protein content by 50%. A 21-nucleotide sequence, 5'-CGCAAGCCGGGTAGCCCGGC-3', was synthesized for use as a no-effect control ODN. In genes showing the closest match to this sequence in GenBank, none had greater than a 14-nucleotide stretch of complementary sequence.

Antisense ODNs were introduced into replicate culture dishes and prepared as described above, using the procedure recommended by the manufacturer. The amount of transfection was estimated by cotransfecting cells with plasmid DNA for green fluorescent protein.
(pXJ40-GFP), which was a gift from Dr. Christine Hall (Institute for Neurology, University College, London, UK) (14). Cells were serum-starved for 1 h in medium without FBS and antibiotics. Antisense agent (2 μg) was diluted into 125 μl of WIHC containing 6% FBS and mixed by inversion, and 4 μl of Lipofectamine PLUS (GIBCO Invitrogen, Grand Island, NY) were added. Each tube was inverted and incubated at 37°C for 20 min. In separate tubes, 6 μl of Lipofectamine reagent was made up to 1 ml with WIHC and inverted. Antisense and reagent solutions were combined, vortexed briefly, and incubated at room temperature for 15 min. The medium on the cells was replaced with this solution. Cells were incubated for 7 h under routine conditions. They were washed twice with culture medium and incubated for up to 48 h while being subjected to additional treatments. In PMA-treated cultures, this procedure was termed “double knockdown,” because knockdown of PKC at the transcriptional level was followed by possible PMA-induced turnover of PKC.

Transfection with small interfering RNAs. Small interfering RNA (siRNA) duplexes were designed to target 21-nt sequences of the respective rat PKC-ε and PKC-γ mRNAs. Unique regions of sequence were found by doing multiple alignment with other PKC isoforms with ClustalX 1.83 software (54) and using BLAST searches in GenBank (http://www.ncbi.nlm.nih.gov) to confirm that there was no homology between the sequence identified and any other sequence in the rat genome. siRNAs targeting DNA strands 5′-AAGACGACCGTCAAACTGACAG-3′ (bases 1212–1232) and 5′-AACCTTCTGACGTCACTGTAC-3′ were purchased from Qiagen (Alameda, CA). A control siRNA duplex supplied by Qiagen, 5′-AAGTACGTTCGCCTACGTAG-3′, was also used.

For siRNA transfection, cells were subcultured into 35-mm wells in multwell dishes and allowed to attach to the dishes, and the siRNAs from Qiagen (Alameda, CA) were incubated for 15 min at room temperature to allow formation of precipitation or stored in liquid nitrogen until use. Polyclonal antibodies (anti-PKC-ε, Lexington, KY) was used. Following the manufacturer’s procedure introduced sources of variability, namely, protein loss through nonprecipitation of antibody complex. Moreover, immunoprecipitation for sequential antibody-binding steps, which were avoided by immunoprecipitation of antibody complex. Moreover, immunoprecipitation followed by gel separation had the additional advantage that the results were subject to Beer’s Law. Assuming that a proper ratio of dye volume to sample concentration is used, Beer’s Law states that optical density is directly proportional to the concentration of material.

Gels were washed in deionized H2O, three times for 15 min per repeat, and stained using Gelcode blue stain (Pierce, Rockford, IL). Gels were rinsed four times for 30 min per repeat. Images of the gels were taken using a Fotodyne digital gel scanner. The immunoprecipitated isoform was quantified by reference to the integrated optical density of molecular weight markers on the same gel. There were two major sources of variability in this procedure. One was that any loss of standard during loading would artificially elevate the apparent level of PKC in the sample. The number of marker bands available for standardization also varied, because the faster running bands at 66 and 55 kDa were sometimes invisible due to diffusion. Because the marker proteins varied in dye-binding capacity, this increased the variability of the reference lane. Within these constraints, quantitation appeared to be highly reproducible for bands containing >100 ng.

To determine whether accurate predictions could be based on the knowledge of how much protein remained after PMA-induced turnover, we checked the PKC content in samples exposed to the double knockdown protocol (transcriptional inhibition followed by PMA-induced PKC-ε degradation). The final content of PKC was estimated using half-life values of 24 h up to 5 h of PMA exposure, and 48 min thereafter (see Fig. 1 and Ref. 58) in the standard equation for half-life:

$$\log \left(\frac{N_i}{N_f}\right) = 0.301(\text{th})$$

where \(N_i\) is the amount of PKC-ε found in untreated cells, \(N_f\) is the amount remaining, \(t\) is the time of decay, and \(h\) is the half-life of the protein.

Counts of stress fiber-containing cells and mathematical manipulations. Cultured cells were fixed with 3% formaldehyde, made up fresh from paraformaldehyde in PBS. A stock of 0.4 μg/ml tetramethylrhodamine isothiocyanate-labeled phalloidin (Sigma-Aldrich) was diluted 1:600 in PBS to stain filamentous actin. Coverslips were mounted on the dishes with ImmunoFluore medium (ICN Biomedicals, Aurora, OH). Because all of the cells contained filamentous actin, it was necessary to define how a robust stress fiber would be distinguished from fine filaments in the assay. Moreover, folds of cytoplasm parallel to the periphery of the 1000 W cells could resemble stress fibers even though their origin differed from that of true fibers. For preparations examined in a Zeiss AxioPhot microscope equipped with a ×40 Plan-Neofluar lens and a rhodamine filter set, positive cells were defined as those showing one or more robust fibers. Positive cells were defined as those showing one or more robust fibers. Counts of stress fiber-containing cells were taken using a Fotodyne digital gel scanner. The immunoprecipitated isoform was quantified by reference to the integrated optical density of molecular weight markers on the same gel. There were two major sources of variability in this procedure. One was that any loss of standard during loading would artificially elevate the apparent level of PKC in the sample. The number of marker bands available for standardization also varied, because the faster running bands at 66 and 55 kDa were sometimes invisible due to diffusion. Because the marker proteins varied in dye-binding capacity, this increased the variability of the reference lane. Within these constraints, quantitation appeared to be highly reproducible for bands containing >100 ng.

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PKC-ε content in cells after PMA treatment appeared to follow a nonlinear decay curve. To fit the relationship between enzyme degradation and the frequency of stress fiber-containing cells, we implemented linear and nonlinear approximation methods with Matlab (The MathWorks; http://www.mathworks.com) and Fathom software (Key Curriculum Press; http://www.keypress.com).

Statistical treatment of data. Data on isozyme content were subjected to a general linear model (GLM) procedure, implemented in Minitab software. The GLM was used because of the unbalanced numbers of samples recorded for each isozyme. For counts of stress fiber-containing cells after double knockdown by ODN transfection, the statistical significance of differences in time and agents was evaluated using GLM. One-way ANOVA was used to test the time-dependent differences. Agent-dependent differences were evaluated using the two-sample t-test. The Student’s t-test was used to define the 95% confidence intervals around the means that showed obvious differences in isozyme content or stress fiber frequency (13).

Real-time PCR. RNA was isolated using an Absolutely RNA kit (Stratagene, La Jolla, CA). For the real-time reaction, 2 μg of RNA treated with DNase I (Invitrogen-Life Technologies, Grand Island, NY) were combined with 1 μg of random decamer primers (Ambion, Austin, TX) in nuclease-free water and heated to 70°C for 3 min and then placed on ice. To this mix, we added 0.5 mM of each deoxynucleotide (dNTP), 10 mM DTT, 30 units of Prime RNase inhibitor (Eppendorf, Westbury, NY), 200 units of Moloney murine leukemia virus reverse transcriptase (RT; Invitrogen-Life Technologies), PCR buffer (10 mM Tris·HCl, pH 8.3, 50 mM KCl, and 1.5 mM MgCl₂) and nuclease-free water in a final volume of 20 μL. This was incubated at 37°C for 1 h, and then the enzymes were heat inactivated for 10 min at 92°C. A negative control reaction was performed without the RT to determine whether there was genomic DNA contamination. Only data showing no such contamination were used.

The rat PKC-ε primers used for real-time PCR were forward 5′-CCCCTTGCTACAGGACAAT-3′ and reverse 5′-AGCTGCCATCATGATACCA-3′ (bases 1242–1350, GenBank accession no. M18331), and the internal TaqMan probe was 6-FAM-ATCCGGAAGGCTTTGCAT-TAMRA (bases 1280–1298; Qiagen). Reactions were run on a Cepheid SmartCycler (Sunnyvale, CA). Final concentrations were 200 μM dNTPs, 0.3 μM gene-specific primers, 0.2 μM TaqMan probe, 4 mM magnesium acetate, 2 μL of cDNA, and 1.5 units of MasterTaq (Eppendorf) and MasterTaq buffer in a final volume of 25 μL. The annealing temperature was 58°C. TaqMan 18S rRNA detection reagents (Eurogenics, San Diego, CA) were used for normalization of the data according to the manufacturer’s instructions.

RESULTS

Relationship between PKC degradation and actin-based features. To determine whether the changes in actin architecture were correlated with PKC content, we determined which, if any, isozymes were decreased over the time course following PMA exposure. The amount of each isozyme in the 1000 W cells was determined by immunoprecipitation, followed by SDS-PAGE. PKC-α and PKC-ε were found to be present at the highest levels of the seven isozymes found in the cells. Samples at time 0 and 30 min contained averages of 0.70 and 1.43 μg PKC-α per sample, respectively. Because a real increase in content was unlikely to occur within 30 min, the true starting content was assumed to be ~1.1 μg per 3 × 10⁵ cells. Therefore, the α-isozyme was grouped into one class with the ε-isozyme, which had 1.7 μg/sample, based on their prevalence. The γ-isozyme was also prevalent at 0.99 μg/sample (Fig. 1). Analysis of the content of PKC-α over the time course did not suggest degradation, because none of the mean values differed substantially (Fig. 1A). In contrast, PKC-ε was reduced to 38% of its original content by the 15-h interval (Fig. 1B).

Statistical tests on the above data showed that the content varied among the isozymes at a significance level of P < 0.001. No statistical difference was evident in the overall pooled time data, however, and there was no interaction between the two variables (Table 1). Because the PKC-ε seemed

<table>
<thead>
<tr>
<th>Table 1. P values for differences in isozyme content vs. time in two-sample t-test</th>
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<tbody>
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<td>Source</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>Time</td>
</tr>
<tr>
<td>Isozyme</td>
</tr>
<tr>
<td>Interaction</td>
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</tbody>
</table>

P value calculations correspond to data shown in Figs. 1 and 2. SS, sum of squares; MS, mean squares.
to be degraded rapidly between 5 and 10 h after PMA treatment, content values of early times and late times were pooled and tested for a significant difference. The P value of the difference was \( P = 0.001 \) (T-value 4.01). Tests of the PKC-\( \epsilon \) content with Student’s \( t \)-test also revealed that the content in the 5- and 15-h samples differed (confidence intervals: 1.84 < 2.24 < 2.64 vs. 0.999 < 0.644 < 1.20). PKC-\( \gamma \) content also underwent a gradual decline to ~25% of the starting value by the end of the time course, consistent with net degradation (Fig. 1C). However, \( t \)-tests on PKC-\( \gamma \) content values indicated no significant difference over the time course.

Of the isozymes that were lower in content in cells, PKC-\( \beta \) declined within 2 h after PMA, remained low through the 10-h interval, and then rose again at the end of the time course. The results of \( t \)-tests indicated that the content at 2 h (95% confidence interval: 0.22 < 0.24 < 0.25) was lower than at time 0 (0.31 < 0.39 < 0.48). The increase in content at 15 h may have been related to the well-known PMA-initiated transcriptional upregulation of the gene (39). The \( \eta \), \( \delta \), and \( \zeta \)-isozymes failed to show any sign of degradation in PMA-exposed cells. As a member of the aPKC group, PKC-\( \zeta \) would not be expected to be activated or degraded. However, its content underwent an anomalous rise over the time course (Fig. 2). With the exception of PKC-\( \eta \) samples, in which a secondary antibody was used to recover the protein, no bands other than PKC were found in the immunoprecipitates (the online version of this article has supplemental data; see supplemental Figs. 1 and 2). These results suggested that the \( \epsilon \) - and \( \beta \)-isozymes underwent significant degradation after PMA exposure.

The possibility that the switch between actin-based phenotypes was dependent on degradation of one of these PKC isoforms was explored. This was done by determining the frequency of stress fibers after knockdown of each isozyme at the transcriptional level with antisense ODN. The appearance of stress fiber-containing cells was similar to that shown for the siRNA knockdown treatments (see below). A two-way ANOVA test was used to determine whether there was an interaction between time and treatment. The interaction between time and treatment was significant at \( P < 0.001 \) (Table 2). From pooled data of all treatments, we found that stress fiber frequency was elevated significantly at 10–15 h compared with the earlier times (Table 3). Within each treatment group, the amount of stress fibers present over the time course was enhanced by knockdown with any specific ODN compared with control (Table 3). Samples transfected with ODN against PKC-\( \beta \) isozyme, however, showed no significant difference in stress fiber frequency compared with the control at comparable time intervals (Fig. 3).

In cells transfected with ODN against PKC-\( \epsilon \) DNA, the frequency of stress fiber-containing cells was high at the beginning of the time course. These cells differed significantly from control, as illustrated by comparing the 95% confidence interval around the respective means (28.5 < 31.3 < 34.2 vs. 10.9 < 12.3 < 13.8). Double-knockdown cells that were treated with PMA for 15 h also showed a significant increase in stress fibers compared with control, because 68% of the cells had stress fibers compared with 23% in the control (confidence intervals: 55.8 < 68.3 < 80.8 vs. 8.32 < 22.7 < 31.0). As previously noted in studies of PMA-treated cells, the level of stress fibers reached a low point at 2 h (21). This difference was not significant in the analysis of pooled data (Table 3), but in cells transfected with PKC-\( \epsilon \) ODN, the depression of stress fiber frequency appeared significant. The 2- and 10-h mean percentages, represented by 95% confidence intervals, were 7.0 < 16.0 < 25.0 vs. 31.9 < 40.7 < 49.4. Therefore, fiber-containing cells increased progressively from 2 h to an approximately fourfold higher level by 15 h in cells treated

Table 2. \( P \) values for differences in stress fiber counts vs. time or specific ODN in two-way ANOVA

<table>
<thead>
<tr>
<th>Source</th>
<th>Degrees of Freedom</th>
<th>Adjusted SS</th>
<th>Adjusted MS</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time</td>
<td>4</td>
<td>7194.4</td>
<td>1798.6</td>
<td>42.9</td>
<td>0.000</td>
</tr>
<tr>
<td>ODN</td>
<td>3</td>
<td>1511.1</td>
<td>503.7</td>
<td>12.0</td>
<td>0.000</td>
</tr>
<tr>
<td>Interaction</td>
<td>12</td>
<td>3681.6</td>
<td>306.8</td>
<td>7.32</td>
<td>0.000</td>
</tr>
</tbody>
</table>

\( P \) value calculations correspond to data shown in Fig. 3. ODN, oligodeoxynucleotide.
with ODN against PKC-ε (Fig. 3). Stress fiber-containing cells also rose dramatically in cells transfected with ODN against PKC-α, but these values did not differ significantly from either controls or knockdown cells transfected with antisense ODN against PKC-α at earlier time points (data not shown).

The finding that the stress fibers were elaborated whenever the PKC-ε was knocked down indicated a negative relationship between the two variables. This suggested that PKC-ε turned over stress fibers in untreated cells. We reasoned that general inhibitors of transcription, such as amanitin and actinomycin D, might block synthesis of new molecules sufficient to evaluate the effect of inhibition of protein synthesis on stress fiber production. When the effect of these inhibitors on the content of specific isozymes was evaluated after 10- and 15-h exposures, however, the amount of isozyme recovered was similar to that found for control cells not treated with the inhibitor (Li Y, unpublished results). The results confirmed that the half-life of these proteins was longer than 15 h (see Introduction).

Fit of inverse relationship between PKC-ε and stress fibers.

To gain insight into the mechanism of PMA-induced remodeling of stress fibers, we fitted a mathematical expression to the negative effect of PKC-ε on fiber formation or stability. Data were obtained from two types of experiments. The level of PKC-ε in each cell sample after various intervals of PMA exposure was known from the data of Fig. 1B. Values resulting from PMA-induced degradation were related to the known frequency of stress fiber-containing cells. Additional data were obtained by estimating the amount of PKC-ε that remained after the protocol for double knockdown, i.e., introduction of specific antisense ODN. Amounts were calculated for times 2–15 h after transcription was halted by the antisense agent, assuming a 24-h half-life of the PKC-ε protein. The results were used to derive the following function:

Table 4. Values used to derive parameters in curve-fitting equation

<table>
<thead>
<tr>
<th>Variable X</th>
<th>Variable Y</th>
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<tbody>
<tr>
<td>PKC-ε, μg</td>
<td>Cells forming stress fibers, %</td>
</tr>
<tr>
<td>0.39</td>
<td>68.3 (15 h)</td>
</tr>
<tr>
<td>0.76</td>
<td>40.7 (10 h)</td>
</tr>
<tr>
<td>0.73 (15 h*)</td>
<td>32.5</td>
</tr>
<tr>
<td>1.25 (10 h*)</td>
<td>32.5</td>
</tr>
<tr>
<td>1.37</td>
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<tr>
<td>1.58</td>
<td>26.3 (5 h)</td>
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<td>1.68 (0 h*)</td>
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<td>1.74 (1/2 h*)</td>
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<tr>
<td>2.24 (5 h*)</td>
<td>34.5</td>
</tr>
<tr>
<td>2.33 (2 h*)</td>
<td>20.5</td>
</tr>
</tbody>
</table>

Asterisks indicate numbers derived from experiments on posttranslational (single) knockdown.

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Mean %Stress Fiber-Containing Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.9 (-----•-----)</td>
</tr>
<tr>
<td>10</td>
<td>37.6 (-----•-----)</td>
</tr>
<tr>
<td>15</td>
<td>49.1 (-----•-----)</td>
</tr>
<tr>
<td>2</td>
<td>21.0 (-----•-----)</td>
</tr>
<tr>
<td>5</td>
<td>23.1 (-----•-----)</td>
</tr>
<tr>
<td>Mean</td>
<td>24.0</td>
</tr>
</tbody>
</table>

Table 3. 95% confidence intervals for stress fiber-containing cell means varying with time and treatment, based on pooled standard deviations

<table>
<thead>
<tr>
<th>ODN</th>
<th>Mean %Stress Fiber-Containing Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-ε</td>
<td>36.5 (-----•-----)</td>
</tr>
<tr>
<td>Anti-α</td>
<td>31.3 (-----•-----)</td>
</tr>
<tr>
<td>Anti-β</td>
<td>32.4 (-----•-----)</td>
</tr>
<tr>
<td>Anti-random</td>
<td>22.7 (-----•-----)</td>
</tr>
<tr>
<td>Mean</td>
<td>24.0</td>
</tr>
</tbody>
</table>

95% confidence interval calculations correspond to data shown in Fig. 3 and Table 2. Fiber-containing cells in pooled 10- and 15-h data differed significantly from the pooled 0-, 2-, and 5-h data. Stress fiber-containing cells in each treatment group differed significantly from the control ODN group.

Fig. 3. Effect of isozyme knockdown on prevalence of stress fibers. The prevalence of fibers was similar for cells transfected with control oligodeoxynucleotide (ODN) against random sequence and against PKC-β. At 0, 10, and 15 h of PMA exposure, fiber prevalence was enhanced in samples transfected with antisense ODN directed against PKC-ε. PKC-α knockdown changed the apparent frequency of stress fiber-containing cells at 10 and 15 h. Error bars represent SD. Raw data are shown in Supplemental Data Table 1.
where \( F \) is the frequency of stress fiber-containing cells and \( x \) is the PKC content.

Using linear approximation methods in Matlab, we obtained the function

\[
F(x) = F_0 e^{-\alpha(x-x_0)}
\]

\( F_0 \) is the PKC content.

For nonlinear approximation, we wanted to find the value of

\[
F(x) = 0.3806 e^{-0.3051(x-0.387)}
\]

(1)

Two functions could be obtained in Fathom, depending on whether the intercept was locked. With the intercept locked at 0:

\[
F(x) = 0.6833 e^{-0.727(x-0.387)}
\]

(2)

With the intercept not locked:

\[
F(x) = 0.3788 e^{-0.305(x-0.387)}
\]

(3)

For nonlinear approximation, we wanted to find the value of the least-squares:

\[
\min_a \sum_{i=1}^{n} [y_i - f(a) e^{-a(x_i-x_0)}]^2
\]

Finding the first derivative of the above equal to 0:

\[
g(a) = \sum_{i=1}^{n} [y_i - f(a) e^{-a(x_i-x_0)}] e^{-a(x_i-x_0)} (x_i - x_0) = 0
\]

We used the bisection algorithm to find a solution to the above continuous function \( g(a) \) on the interval \([0.84, 0.85]\), where \( g(0.84) \) and \( g(0.85) \) have opposite signs. Thus we obtained the final approximation function:

\[
F(x) = 0.6833 e^{-0.8412(x-0.387)}
\]

(4)

A comparison between the above functions and the original data is shown in Fig. 4. Despite the wide spread of the experimental data, it was clear that the best fits to the data were the Fathom linear function corresponding to Eq. 2 and the least-squares function corresponding to Eq. 4.

Knockdown by siRNA and transcript quantification. To confirm that the subject of the double knockdown procedure was in fact PKC-\( \epsilon \), and not a gene with homology to the sequence targeted by the ODN, we tested the effects of knockdown using an inhibitory RNA targeted to a different portion of the PKC-\( \epsilon \) sequence. In cells treated by siRNA-mediated knockdown of PKC-\( \epsilon \) and then exposed to PMA, stress fibers were more prevalent throughout the time course than had been observed with the ODN knockdown. The induction of stress fibers was also less than that observed with ODN knockdown, because the frequency of stress fiber-containing cells at the end of the time course was 0.52 compared with 0.68 (Fig. 5). However, time-dependent differences in the pooled data were significant at the level \( P = 0.01 \). The difference between the data from PKC-\( \epsilon \) siRNA treatment and control siRNA was significant at \( P < 0.001 \) (Table 5). In addition, obvious differences in stress fiber organization were observed after the two types of treatment. Fibers tended to cross the center of the PMA-exposed cells and, occasionally, to radiate from a central point or form shorter parallel arrays (Fig. 6). Thus knockdown results confirmed that 1) a shortage of PKC-\( \epsilon \) was correlated with stress fiber formation, and 2) more fiber formation occurred both after transcriptional downregulation (time 0) and after PMA-initiated degradation relative to the comparable control cells.

The above-described data indicated that PKC-\( \epsilon \) was the only isozyme that underwent significant knockdown by PMA and

![Fig. 4. Mathematical fit of relationship between PKC-\( \epsilon \) content and frequency of stress fiber-containing cells. Data values are indicated (*), along with curves representing Fathom linear approximation corresponding to Eq. 2 (+) and Matlab linear approximation (−). The best fit to the data was obtained with Fathom (Eq. 2) and the nonlinear approximation of Eq. 4 (−). Matlab linear approximation was a poor fit to the data at both extremes of the curve.](http://ajpcell.physiology.org/)

![Fig. 5. Effect of PKC-\( \epsilon \) knockdown on prevalence of stress fibers. Transfection of cells with small interfering RNA (siRNA) against PKC-\( \epsilon \) caused initially higher prevalence, which decreased from time 0 and then increased again. The prevalence of fibers in cells transfected with siRNA against random sequence DNA resembled that observed in control samples treated with PMA alone (18). Error bars represent SD. Raw data are shown in Supplemental Data Table 2.](http://ajpcell.physiology.org/)
affected the integrity of stress fibers. To better understand this effect, we wanted to determine experimentally values that would test the mathematical expression previously fit to the data. First, we determined the amount of transcript left after knockdown by PKC-ε siRNA. Preparations of PKC-ε transcripts were made after introduction of the specific and control siRNAs. When cDNAs were selected for amplification and assay by real-time PCR, the samples showed C_T (cycle time) values of 27.86 for single knockdown, 27.87 for random sequence knockdown, and 29.09 for double-knockdown treatments (Fig. 7A). PCR graphs of PKC-ε mRNA levels were compared in each case to the level of an internal standard for total cellular material by amplifying 18S ribosomal RNA. C_T values for the latter were 11.98, 12.13, and 11.98, respectively. This indicated that the amount of ribosomal RNA was nearly identical in all samples (Fig. 7B). Because C_T increases when there is less mRNA to be amplified, the increased value after transcriptional knockdown of PKC-ε showed that about one-half of the transcript remained. PMA treatment itself had no effect on the amount of PKC-ε mRNA (Fig. 7).

Predictions from mathematical fit and validation. The mathematical expression for the inverse relationship between PKC-ε and stress fiber formation allowed us to obtain quantitative estimates of PKC-ε turnover under various conditions. When the amount of transcript left after the siRNA-induced knockdown procedure was known, we could predict the amount of PKC-ε protein that should remain after double knockdown. The calculations were simplified by assuming that transcript was reduced 8 h after introduction of siRNA against PKC-ε. We then solved for the amount of protein remaining after the number of elapsed half-lives (see MATERIALS AND METHODS). In the case of PMA-induced degradation alone, a half-life of 24 h was assumed up to 6 h of PMA exposure. Thereafter, the value of 48 min was assumed for the half-life in conformity with data of Fig. 1B. After transcript ablation alone, the estimated decrease in PKC-ε by the time of PMA treatment was 0.14 μg/sample. By 6 h after PMA exposure, the additional estimated loss was 0.12 μg, bringing the PKC-ε content down to 1.45 μg/sample. Thereafter, rapid loss of the protein by PMA-induced turnover would result in 0.75 μg/sample at the 10-h time point, when the assumption was tested (prediction I).

The residual protein content left after double knockdown could be predicted on the basis of frequency of stress fiber-containing cells, using Eq. 4 (prediction II). Stress fiber-containing cells were determined in the same experiment in which the amount of PKC-ε was measured using the immunoprecipitation method described above (Figs. 1 and 2). Predictions obtained using both methods I and II were tested by comparing the end points within a single experiment. Samples treated with PMA alone had slightly less protein throughout the time course than was found in previous experiments, by ~50% (Table 6). Samples transfected with control ODN and siRNA agents showed values closer to the predictions than the PKC-ε knockdown samples. They showed 62 and 79%, respectively, of the predicted content, which was close to the 52% of PKC-ε that remained in the PMA-treated control after 10 h. In samples transfected with agents against PKC-ε (ODN or siRNA), the experimentally determined amounts were less than predicted. They were 28 and 30%, respectively, of the levels predicted using method I. This suggested that the predictive model overestimated the amount of protein that would remain after
double knockdown. The simplest explanation for this discrepancy was that the amount of transcript remaining was less than the 50% estimated by the PCR technique.

The goodness of fit of the experimental data to the half-life calculations confirmed that the knockdown of the PKC-ε content was predictable. Additional calculations based on Eq. 4 could be used to predict the protein content based on the frequency of stress fibers. Whether double knockdown employed a control sequence unrelated to PKC-ε or the PKC-ε sequence itself, experimental values ranged ~25% of values estimated from stress fiber levels (Table 6). This suggested that there was a tendency for the PKC-ε content to be overestimated based on these levels. This was true even though PKC-ε content and stress fiber counts were analyzed within the same experiment (Table 6). The results suggested that the equation (Eq. 4) showed adequate predictive power but should be revised to assure a better fit at high stress fiber levels.

**DISCUSSION**

**PKC's role in the transformed shape phenotype.** Previous studies in this laboratory had the objective of finding functional changes that correspond to transformation-dependent changes in cell shape. The latter were defined by mathematical methods, which provided the advantage that each cell could be classified precisely with regard to its normal or cancer-type phenotype (17). This approach not only enhanced our understanding of physiological differences between normal and cancer cells (18, 20, 21, 44) but offered the opportunity to explore features of the phenotype further. With the use of a sophisticated classification technique based on latent factor extraction, certain morphometric features called factors could be linked to cell specializations. Factors that were altered in transformed cells represented filopodia, rounding up of the whole cell, and PAK-dependent adhesive structures specializations, as well as coarse structures in the upper part of the cell and factors related to vesicle trafficking (size of projections on either of the upper contours, variance in size of projections in the second contour, and others). The finding underlying the present research was that values of many of these factors were coordinately affected by PMA treatment. At the same time, PMA caused changes in actin-based features, stimulating a phase of high ruffling activity followed by stress fiber formation. Most of the factors’ values that were altered by PMA exposure were reversed by 10 h.

One of the questions raised by the earlier findings is how these phenotypic changes are regulated in concert. Because the major cellular receptor for PMA is PKC, and diverse isozymes of PKC were known to be degraded after PMA exposure (see Introduction), the activation and subsequent degradation of PKC offered a facile explanation for changes in the phenotype. Of the isozymes present in 1000 W cells (PKC-α, -β, -γ, -δ, -ε, -η,  and -ζ), only a few were activated by PMA exposure and consequently declined in content. PKC-β, -ε, and possibly -γ were among those that were activated. Of these, only PKC-ε could be related to turnover of stress fibers. PKC-β was ruled out because it was not altered by PMA exposure.

**Table 6. PKC-ε content predicted for knockdown procedures compared with experimental amount**

<table>
<thead>
<tr>
<th>Knockdown Treatment</th>
<th>Mean Frequency of Stress Fibers</th>
<th>Experimental Value, µg</th>
<th>Predicted Value (I), µg</th>
<th>Predicted Value (II), µg</th>
<th>Experimental (I) PKC-ε, %</th>
<th>Experimental (II) PKC-ε, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>PMA alone 10 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Double</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control ODN</td>
<td>0.33</td>
<td>0.29</td>
<td>0.47</td>
<td>0.47</td>
<td>1.24</td>
<td>62</td>
</tr>
<tr>
<td>PKC-ε ODN</td>
<td>0.68</td>
<td>0.12</td>
<td>0.40</td>
<td>0.39</td>
<td>1.54</td>
<td>79</td>
</tr>
<tr>
<td>Control siRNA</td>
<td>0.26</td>
<td>0.37</td>
<td>0.47</td>
<td>0.54</td>
<td>0.65</td>
<td>28</td>
</tr>
<tr>
<td>PKC-ε siRNA</td>
<td>0.55</td>
<td>0.11</td>
<td>0.40</td>
<td>0.54</td>
<td>1.54</td>
<td>79</td>
</tr>
</tbody>
</table>

The content of PKC-ε remaining after double knockdown was predicted based on the estimated half-life of the molecules (prediction I), as detailed in MATERIALS AND METHODS. PKC-ε content was predicted based on the frequency of stress fibers (Eq. 4) in samples treated by double knockdown with ODN and siRNA (prediction II). Less PKC-ε was recovered experimentally from the samples treated by transcriptional knockdown or sham knockdown than had been predicted on the basis of stress fiber production. Asterisks indicate experimental data from PMA-induced PKC-ε degradation (Fig. 3) used for comparison.
out by the data, because its content declined early in the time course and later rose again. These were periods when stress fibers were decreased and increased, respectively. When the role of PKC-γ was explored by introducing PKC-γ-specific siRNA, fiber formation was indistinguishable from that of control samples. This made a role for PKC-γ in stress fiber dissolution unlikely (Li Y and Plummer H, unpublished results). Only one isozyme remained, namely, PKC-ε, whose activation could be related to phenotypic changes following PMA exposure. Of course, additional isozymes may have been activated but not degraded.

PKC-ε binding is bifunctional, as one site binds to actin and another to receptor for activated C kinase (RACK). In glioma cells, PKC-ε was found to be associated with integrin and RACK at focal adhesions (2), and it was cotransported with endocytosed integrin. PKC-ε dissociated from integrin-containing vesicles in a way that depended on phosphorylated vimentin (24). Consistent with its role in vesicular trafficking, PKC-ε binds to coatomer protein, β′-COP (10). In previous studies of PKC-ε biochemistry, investigators mentioned that overexpression of a construct of PKC-ε with the actin-binding site deleted prevented ruffling stimulation and stress fiber dissolution (46). The current data support the latter conclusion but reject a role for PKC-ε in ruffling, because neither the ODN nor the siRNA inhibitors of PKC-ε transcription suppressed PMA-induced ruffling (Urban J and Li Y, unpublished results). Another bifunctional molecule, α-actinin, has one site that binds to actin and another that binds the cytoplasmic domain of the α1-subunit of integrin. Introduction of the portion with actin-binding domain deleted caused dissolution of the stress fibers, an effect that was attributed to competition at the integrin-binding site (42). This study provides a precedent for an actin-binding structural protein participating in the organization of the stress fiber at adhesive sites.

Although current results suggested a role for PKC-ε in modulating actin fibers or their attachment sites, the only reported localization to stress fibers has been in cardiac muscle cells (33). PKC-ε in 1000 W cells was found on vesicle-like structures and circular profiles clustered around the nuclear envelope, as previously described for other epithelial cells (32). Although these sites reflect the vesicle-trafficking function of PKC-ε, they are not obviously related to stress fibers. Moreover, localizations conducted in cells with and without added PMA failed to reveal obvious differences (Heckman C, unpublished data). Therefore, the mechanism of stress fiber turnover could be indirect and take place through PKC-ε-mediated activation of another enzyme or even through transcriptional up- or downregulation of a new gene product. A third possibility is that PKC-ε works via a “hit-and-run” mechanism. Preliminary evidence indicates that novel phosphorylated substrates may be found in the 1000 W cells corresponding to proteins involved in the direct PKC-ε-mediated turnover of stress fibers (30). Using the current method to ablate PKC-ε, we sought peptides from the double-knockdown cells that matched phosphopeptides from control cells by using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Thus there is also a possibility that PKC-ε directly affects the steady-state dissolution of stress fibers.

Effects of PKC on actin superstructure in cultured cells. Laboratories studying muscle cell physiology describe a variety of known PKC-mediated reactions that can promote the formation or stability of the stress fiber. Such pathways include one in which PKC phosphorylates the myosin phosphatase target subunit 1 (MYPT1). MYPT1 targets phosphatase to myosin, and PKC-mediated phosphorylation reduces its affinity for both phosphatase and myosin. Because myosin light chain (MLC) kinase leads to MLC phosphorylation, which recruits myosin to actin, this activity persists in the absence of phosphatase. Thus this mechanism enhances stress fiber assembly by driving myosin II binding on actin (53, 56). Another mechanism of increasing filament contractility involves calponin, which inhibits actin-activated myosin ATPase activity when not phosphorylated on Ser175. If phosphorylated on this site by PKC, calponin permits actin-myoosin interaction and muscle contraction (25). This is a physiological mechanism of Ca2+-independent contraction in vascular smooth muscle (22).

Certain PKC-mediated phosphorylation reactions cause depolymerization of the same highly cross-linked actin structures. Bogatcheva et al. (4) observed that although PMA stimulated an increase in MLC phosphorylation, the kinase lacked specificity for sites that promote actin-myosin binding. In lung endothelial cells, PKC phosphorylates MLC at sites Ser1, Ser8 and Thr8. The PMA-mediated increase in activity occurred in conjunction with transient stress fiber destabilization and rearrangement of peripheral actin, which was followed by sustained MLC dephosphorylation at Thr18 and rearrangement of actin fibers into a gridlike network (4). Likewise, phosphorylation of the myristoylated alanine-rich C kinase substrate (MARCKS) and fascin dramatically decreased the ability of these proteins to cross-link actin filaments (11, 59). Actin filament cross-linking is transiently reduced in the PMA-treated tracheal cells used in the current studies, making these depolymerization mechanisms of particular interest. However, the different subsets of isoforms and types of substrates in the cells may dictate varied consequences of phorbol ester-mediated PKC activation in different cell types.

As illustrated by the above examples, PKC can participate in a pathway actively by its kinase activity. Another aspect of the problem, less often studied, is how PKC can affect a pathway passively by recruiting molecules to a scaffold. PKC-α is localized to focal adhesion sites by binding to syndecan-4 (31), and it helps drive β1-integrin exposure on the cell surface (36). The existence of these different mechanisms has one unfortunate implication, i.e., the mere knowledge that a specific molecular pathway is operative in a cell provides little assurance that the dominant effect on the cytoskeleton is in response to that pathway. Quantifying features by morphometric methods and fitting data to mathematical equations are two approaches that may help workers to interpret molecular changes in terms of their physiological consequences.

Effects specific to PKC-ε. The isoforms that bind actin directly are logical candidates for regulating actin architecture. A COOH-terminal portion of PKC-BII interacts with actin and causes enzyme autophasorylation (3). The nonhomologous actin-binding site on PKC-ε associates with actin filaments in response to extracellular stimuli in a manner independent of phosphatidyserine (23, 46, 47, 60). The linkage between PKC-ε and actin is strengthened by several findings made in the current studies. First, transcriptional knockdown of the enzyme profoundly affects the frequency of stress fiber-containing cells, even enhancing their frequency before PMA-induced enzyme degradation. Second, there were unusual ar-
rangement of stress fibers after double knockdown of the PKC-ε isozyme, namely, the number and diversity of isozymes involved in signal transduction. Content differences were a favorable subject for investigation, because they were not rapidly reversible like the activation-inactivation cycle of the enzyme. In the current experiments, both the immunoprecipitation and real-time PCR results indicated that the gene-silencing treatment was effective. The finding that there were also significant differences in stress fiber frequency indicated that the majority of cells were affected by the transcriptional inhibitor. Because several isozymes may be activated by a single receptor-ligand binding event, such knockdown methods may be helpful in dissecting out the effects due to one isozyme.

As mentioned above, many regulatory problems could be addressed by linking a specific physiological function to a specific PKC isozyme. In practice, it has been difficult to draw such relationships, because the signaling network in which PKC operates is complex and intersects with upstream regulation of the Rho family GTPases, which also govern actin architecture (6). Although the siRNA technology may knock down transcription of specified isozymes, it is difficult to prevent phosphorylation of substrates by the remaining isozymes, namely, the number and diversity of isozymes in the cytoplasm. In the current research, a double-knockdown approach was used, combined with a predictive equation for relating PKC-ε to stress fibers. The mathematical expression not only facilitated the current studies but will be valuable for future research as well; e.g., to determine whether the potential PKC substrates that are actin-binding proteins (MYPT1, MLC, fascin, or MARCKS) are implicated in stress fiber dissolution in 1000 W cells.

In light of the difficulty of addressing the role of dynamic multiprotein complexes in regulating complex physiological endpoints, the concepts developed in the current studies may be necessary to clarify how PKC-ε affects shape change and motile functions. Because phenotype reversal has proved to be correlated with the therapeutic efficacy of cancer treatments (18), future studies on mechanisms are expected to be relevant to tumor promotion and cancer cell motility. This in turn will improve our understanding of how PKC-ε causes the aggressive and metastatic cancer cell phenotype in breast, colon, and astroglial tumors (41, 43, 51).

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GRANTS

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REFERENCES


30. Lim ST, Longley RL, Couchman JR, and Woods A. Direct binding of syndecan-4 cytoplasmic domain to the catalytic domain of protein kinase \( \alpha \) (PKC\( \alpha \)) increases focal adhesion localization of PKC\( \alpha \). J Biol Chem 278: 13795–13802, 2003.


