Effect of α-Protein Kinase C Neutralizing Antibodies and the Pseudosubstrate Peptide on Phosphorylation, Migration, and Growth of REF52 Cells

Lan Liao and Susan Jaken

The W. Alton Jones Cell Science Center, Lake Placid, New York 12946

Abstract

REF52 cells are a line of rat embryo fibroblasts that express α-, β-, γ-, and θ-protein kinase Cs (PKCs). In this report, we have used neutralizing antibodies to α-PKC to study the role of this specific PKC isoform in REF52 cell functions. Effects of the more general PKC inhibitor, the pseudosubstrate peptide, were also studied. Previous work demonstrated that α-PKC is concentrated in focal contacts of REF52 cells (Jaken, S., Leach, K., and Klauck, T. J. Cell Biol., 109: 697–704, 1989). α-PKC redistributed to the leading lamellipodia of cells stimulated to migrate into an artificial wound, indicating that α-PKC activation may be coupled to migratory stimuli. The effects of the α-PKC neutralizing antibodies and the pseudosubstrate peptide on responses associated with focal contact functions, namely attachment, migration, and growth, were studied. The data demonstrate that the antibodies and the pseudosubstrate peptide were all efficient inhibitors of phorbol ester-stimulated phosphorylation. However, only the pseudosubstrate peptide efficiently inhibited the migration and growth responses necessary to repopulate an artificial wound. These results indicate that PKCs, but probably not α-PKC in particular, are important in these responses. However, because the neutralizing antibodies did not completely inhibit phorbol 12,13-dibutyrate-stimulated phosphorylation, a potential role for α-PKC in migration and growth cannot be excluded.

Introduction

PKC is a key signal transduction enzyme involved in regulation of cell growth and differentiation (reviewed in Refs. 1–3). PKC is also the major cellular receptor for tumor-promoting phorbol esters. Phorbol esters have been used as specific activators to demonstrate the role of PKC in various cellular processes including cell growth, gene expression, exocytosis, and protein phosphorylation. Inhibitors, such as staurosporine and K252-A, have also been used to infer a role for PKC in certain processes. Although several of these are potent PKC inhibitors, their lack of specificity can complicate interpretation of results (4–6).

Recently, a sequence within the regulatory domain of PKC was shown to be a specific inhibitor of PKC isozymes (7). This autoinhibitory sequence is thought to play a major role in maintaining PKC in an inactive conformation in the absence of activating cofactors. The sequence contains several basic amino acids which are important determinants of PKC phosphorylation sites, but it is missing a serine or threonine residue and therefore cannot be phosphorylated. For this reason, the sequence is known as the pseudosubstrate domain. The pseudosubstrate peptide and analogues have been widely used to study PKC functions in cellular responses (8–10). However, it should be noted that serine-substituted analogues are efficiently phosphorylated by several PKC isozymes (11, 12). Therefore, it is unlikely that pseudosubstrate sequences function as specific isozyme inhibitors.

We have developed several mAbs to α-PKC and characterized their effects on α-PKC activities (13). M4 interacts with the catalytic domain and inhibits catalytic activity in vitro. M9 interacts with the regulatory domain and inhibits [3H]PDBu binding in vitro. Inhibition is competitive with phosphatidyserine, indicating that M9 may interfere with phospholipid binding to α-PKC. Although these neutralizing antibodies have potential utility in defining α-PKC-specific functions, their use is limited due to the fact that they are not cell permeable. In this study, we have used osmotic shock and scrape-loading to introduce the neutralizing antibodies into REF52 cells in order to study their effects on phorbol ester-stimulated phosphorylation and associated biological responses.

Previous studies demonstrated that α-PKC is concentrated in focal contacts of REF52 cells (14). Focal contacts are sites of cell attachment to the substratum (15, 16). In order to assess the potential role of α-PKC in these structures, we studied the effects of the pseudosubstrate peptide and the neutralizing antibodies on cell adhesion and migration, i.e., processes known to be associated with focal contact function.

Results

α-PKC Location in Stationary and Migrating Cells. α-PKC is concentrated in the focal contacts of REF52 cells (14), which suggests that α-PKC may function in cell adhesion and/or migration. To further explore this potential function, cells were induced to migrate by artificial wounding, and α-PKC locations in stationary and migrating cells were compared. In cells that were fixed immediately after scraping, α-PKC was located along stress fibers and in focal contacts (Fig. 1). Within 1 h after scraping, α-PKC accumulated at the leading edges of cells. These areas did not contain filamentous actin detectable by phalloidin staining. In contrast to the distinctive staining pattern

Received 12/8/92; revised 1/19/93; accepted 1/19/93.

1 This work was supported by grants from the Council for Tobacco Research (2375A) and the National Cancer Institute (CA53841 and CA37589).

2 To whom requests for reprints should be addressed, at The W. Alton Jones Cell Science Center, 10 Old Barn Road, Lake Placid, NY 12946.

3 The abbreviations used are: PKC, protein kinase C; kDa, kilodalton(s); mAb, monoclonal antibody; PDBu, phorbol 12,13-dibutyrate; REF52, rat embryo fibroblast 52; lgG, immunoglobulin; PBS, phosphate-buffered saline.
seen in stationary cells, \( \alpha \)-PKC staining was much more diffuse in these cells which were preparing to migrate. Within 4 h, the leading edges of cells were broader, flatter, and still relatively devoid of phalloidin staining. \( \alpha \)-PKC was uniformly distributed at the migrating edges of the cells. After 24 h, the loss of cell-cell contacts was apparent. \( \alpha \)-PKC accumulated in dense plaques in the leading edges. \( \alpha \)-PKC was also found in arrowhead patterns characteristic of focal contacts at the trailing edges of migrating cells. Redistribution of \( \alpha \)-PKC to the leading edges of migrating cells suggests that \( \alpha \)-PKC activation may be involved in migratory signaling.

**Inhibition of PDBu-stimulated Phosphorylation by the PKC Pseudosubstrate Peptide in Digitonin-permeabilized REF52 Cells.** To assess the role of PKCs, and \( \alpha \)-PKC in particular, in cellular responses, we studied the effects of specific PKC inhibitors. First, the ability of these compounds to inhibit PDBu-stimulated phosphorylation was tested. Pseudosubstrate peptide inhibition of PDBu-stimulated (i.e., PKC-mediated) phosphorylation was assessed using a digitonin-permeabilized cell assay (17). PDBu stimulated phosphate incorporation into three heat-soluble proteins of 38, 71, and \( >200 \) kDa (Fig. 2).

Phosphorylation of the \( >200 \) kDa protein was more apparent in the insoluble than the soluble fraction, but it did occur in both compartments (see also Fig. 3). The pseudosubstrate peptide effectively inhibited phosphorylation of all three substrates. In some cases, the pseudosubstrate actually decreased phosphate incorporation in PDBu-stimulated cells below that of unstimulated cells. This suggests that PKCs may be partially activated in the absence of PDBu in this assay protocol.

**Inhibition of PDBu-stimulated Phosphorylation by the Anti-\( \alpha \)-PKC Antibodies M4 and M9.** Unlike the pseudosubstrate peptide, the antibodies are too large to permeate the digitonin-treated cells. Therefore, their effects on protein phosphorylation were assessed in cells preloaded with antibodies by osmotic shock. Introduction of the antibodies was verified by staining with appropriate second antibodies. In general, \( >80\% \) of the cells incorporated antibody (see Fig. 4, below). Two anti-\( \alpha \)-PKC antibodies were tested. M9 recognizes the regulatory domain of \( \alpha \)-PKC and interferes with phospholipid and PDBu binding (13). M4 recognizes the catalytic domain and inhibits enzymatic activity in vitro (13). Non-immune goat IgG fraction was used to control for effects.
of the osmotic shock procedure. Three PDBu-stimulated, heat-soluble phosphoproteins were detected in control cultures that did not undergo the osmotic shock treatment. Introduction of nonimmune goat IgG did not interfere with their phosphorylation (Fig. 3). However, phosphorylation of these proteins was diminished in M9-loaded cells. Although the effect could be observed in both digitonin-soluble and -insoluble fractions, the effect was more pronounced in the digitonin-insoluble fraction (Fig. 3, A and B). A decrease in phosphorylation of the three heat-soluble proteins in the digitonin-insoluble fraction was also observed in M4-loaded cells (Fig. 3D); however, no inhibition of soluble protein phosphorylation was observed in M4-loaded cells (Fig. 3C).

**Phorbol Esters Stimulate Membrane Ruffling in REF52 Cells.** To determine whether neutralizing antibodies could inhibit PKC function in vivo, the effects of PDBu on control and M4 antibody-loaded cells were compared (Fig. 4). To demonstrate the effectiveness of the osmotic shock procedure, antibody-loaded cells were cultured on coverslips. After fixing, they were stained with species-specific secondary antibodies to locate the primary antibody. The nonspecific goat immunoglobulin was diffusely dispersed in the cytoplasm and concentrated in the perinuclear region (Fig. 4A). In contrast, the α-PKC-specific antibody M4 was concentrated in arrowhead patterns characteristic of focal contacts (Fig. 4B). Thus, cells loaded with M4 and stained only with second antibody were not dissimilar from unloaded cells fixed and stained with M4 and second antibody. Treatment with PDBu caused extensive membrane ruffling in both control and M4 antibody-loaded cells, indicating that M4 did not inhibit this PDBu-directed response. PDBu treatment did not influence localization of the control goat antibody. In contrast, PDBu treatment was associated with loss of the discrete focal contact-like staining of M4 and the accumulation of perinuclear staining. The loss of α-PKC focal contact staining is consistent with the PDBu effect on disruption of the actin cytoskeleton observed in the phalloidin-stained samples.

**Effect of the Pseudosubstrate Peptide and Anti-α-PKC Antibodies on Cell Migration.** Localization of α-PKC to leading edges of migratory cells implies a potential functional role of α-PKC in cell migration. To test this possibility, we compared the migration of control and pseudosubstrate or antibody-loaded cells into an artificial wound. Cells were loaded with peptide or antibody by osmotic shock and then plated at high cell density on coverslips. A portion of the monolayer was damaged by scraping, and cells were returned to the incubator to migrate into and repopulate the denuded area. At intervals, coverslips were fixed and stained, and the width of the scraped area was determined by measuring photomicrographs. After 32 h, control cultures had nearly repopulated the scraped area. The width of the scrape decreased by 80% (Fig. 5; Table 1). Neither M4 nor M9 significantly inhibited repopulation. In contrast, the width of the wound remained significantly larger in pseudosubstrate-loaded cells. Repopulation was also inhibited in cells that were pretreated with phorbol esters to downmodulate PKCs (data not shown). HA-1004, which preferentially inhibits cyclic AMP-dependent kinase, did not inhibit repopulation. Thus, the pseudosubstrate peptide, presumably through its interaction with PKC, inhibited either the migration or mitogenesis that is important for refilling the scraped area.

**Effect of PKC Inhibitors on Cell Adhesion and Cell Growth.** Anti-PKC antibodies or the pseudosubstrate peptide were introduced into cells by osmotic shock. Control and osmotically shocked cells were plated into 16-mm wells. At the indicated times, the monolayers were rinsed, and the adherent cell number was determined (Table 2). After 4 h, at which time cells have attached but not yet divided, the number of attached cells was similar for each group. Thus, none of the inhibitors interfered with cell attachment. After 24–48 h, however, a significant decrease in cell number was observed in the pseudosubstrate-loaded cells. No effect on M4- and M9-loaded cells was observed. Thus, the pseudosubstrate peptide or antibody interfered with cell growth, attachment, and/or migration important for repopulating the scraped area.

**Effect of Scrape-loaded Anti-α-PKC Antibodies on Cell Migration.** Although the osmotic shock method effectively introduced antibodies into a large proportion of the cell population, it is possible that the intracellular concentrations were too small to effectively inhibit PKC-dependent processes. We therefore used an alternative technique, scrape-loading, to introduce high concentrations of antibody into a small proportion of the cells. Confluent monolayers on coverslips were overlaid with antibody-containing solutions. A thin strip of the monolayer was scraped, and cells bordering the scraped area...
rapidly accumulated antibodies (Fig. 6). Within 12 h, cells had entered the scraped area and begun to divide. Cultures were fixed and stained with appropriate second antibodies to determine whether cells containing anti-\(\alpha\)-PKC antibodies were preferentially located internal to or at the edge of the wound (Fig. 6). Results were quantitated by measuring the distance of antibody-containing cells from the wound on photomicrographs. After 12 h, 60 ± 4% of the nonimmune goat antibody-containing cells were still near the edge of the wound (within 1 cm on the photomicrographs). Similar results were calculated for M4- and M9-containing cells, with 66 ± 1% and 54 ± 8%, respectively, near the edge of the wound. After 24 h, several antibody-positive cells were still apparent. Mitotic figures could occasionally be found in antibody-positive cells (Fig. 6, arrow). At higher magnification, some antibody-containing cells assumed a morphology characteristic of migrating cells (Fig. 6, bottom panel). Therefore, M4- and M9-containing cells did not migrate more slowly into the wounded area than their neighboring cells.

**Discussion**

PKC has been implicated in a wide variety of physiological processes; however, the lack of specific inhibitors has made it difficult to study the precise role of PKCs in these processes. Cell-permeable inhibitors of PKC, such as K252-A and staurosporine, have been useful, although their lack of specificity can complicate interpretations of results (4–6, 18). Macromolecular inhibitors, such as the pseudosubstrate peptide and the anti-\(\alpha\)-PKC antibodies, are relatively more specific but require special procedures to overcome the cell permeability problem. In these studies, we used three approaches, digitonin permeabilization, osmotic shock, and scrape-loading, to study the effects of these macromolecular PKC inhibitors on phosphorylation and cell function.

---

**Fig. 3.** Inhibition of PDBu-stimulated phosphorylation by anti-\(\alpha\)-PKC-specific monoclonal antibodies. Cells were loaded with nonimmune goat immunoglobulin, the anti-\(\alpha\)-PKC regulatory domain antibody M9 (A and B), or the anti-\(\alpha\)-PKC catalytic domain antibody M4 (C and D) using the osmotic shock procedure described in "Materials and Methods." The ability of PDBu (200 nM) to stimulate phosphorylation in control and antibody-loaded cells was compared using the digitonin-permeabilized cell assay described in "Materials and Methods." Heat-soluble proteins from the digitonin-soluble (A and C) and -insoluble (B and D) fractions were resolved on denaturing polyacrylamide gels, and phosphoproteins were detected by autoradiography. Arrows, positions of the heat-soluble substrates, p38, p71, and p > 200 kDa. Similar results were obtained in 3 independent experiments.
The use of neutralizing, isozyme-specific antibodies as isozyme-selective inhibitors represents a unique approach toward studying isozyme-specific functions. In a previous report, an anti-PKC antibody was shown to inhibit nerve growth factor-stimulated neurite outgrowth in PC12 cells (19). However, the isozyme specificity of this antibody has not yet been defined (20). The recent report that this antibody actually stimulates rather than inhibits PKC catalytic activity confounds interpretation of these experiments (21). We have used α-PKC-specific monoclonal antibodies to both catalytic and regulatory domains (13) to explore the role of α-PKC in cell adhesion and migration. REF52 cells express α-, β-, ε-, and ζ-PKCs, any or all of which may be involved in PKC-dependent processes. Therefore, we have also used the pseudosub-

---

1. Liao and S. Jaken, manuscript in preparation.
substrate peptide, which appears to be an effective inhibitor of all PKC isozymes tested. This assumption is based on the ability of serine-substituted analogues to be efficiently phosphorylated by a variety of PKCs (11, 12). By comparing the effects of the pseudosubstrate peptide and the anti-α-PKC mAbs, the role of α-PKC in specific cellular processes can be investigated. Both the pseudosubstrate peptide and the anti-α-PKC mAbs attenuated phosphorylation of three heat-soluble proteins (p38, p71, and p > 200 kDa). Thus, these three proteins appear to be specific PKC substrates in REF52 cells, and their phosphorylation is at least partially mediated through the α-PKC isozyme.

The three phosphoproteins were recovered in both digitonin-soluble and -insoluble fractions, and the pseudosubstrate peptide attenuated phosphorylation in both fractions. In contrast, the effect of the anti-α-PKC mAbs was more pronounced in the digitonin-insoluble fraction. The incomplete inhibitory activity of the antibodies could be due to many factors, including insufficient concentrations or preferential localization and concentration of the antibodies in the digitonin-insoluble fraction, possibly due to binding to cytoskeletal-associated α-PKC.

We have previously demonstrated that α-PKC is concentrated in focal contacts of REF52 cells, suggesting that it may play a role in focal contact functions such as cell adhesion and migration (14, 22). Furthermore, α-PKC was shown to preferentially accumulate in the leading edges of migratory cells, suggesting that it may function in cell migration (Fig. 1). Therefore, we studied the effects of macromolecular PKC inhibitors on REF52 cell adhesion and migration. Neither the pseudosubstrate peptide nor the anti-α-PKC mAbs inhibited cell attachment. Despite the evidence that the antibodies were efficiently incorporated and properly located (Fig. 4), no inhibition of PDBu-mediated cell ruffling, migration, or growth was detected. Under similar conditions, partial inhibition of phosphorylation could be detected (Fig. 3), indicating that effective intracellular concentrations of inhibitory activity were achieved. However, because inhibition of phosphorylation of soluble substrates was not detected (Fig. 3), we cannot rule out a potential role of α-PKC in cell growth and migration. The pseudosubstrate did inhibit repopulation of the scraped area (Fig. 5) and cell growth (Table 2). Overall, these data indicate that PKC isozymes, although probably not α-PKC, are important for the cell migration and/or the mitogenesis necessary for repopulation of the artificial wound.

Materials and Methods

Materials. PDBu, digitonin, and ATP were obtained from Sigma (St. Louis, MO). [32P]ATP was obtained from New England Nuclear (Boston, MA). The PKC pseudosubstrate peptide (α-PKC 19-31) was synthesized at The W. Alton Jones Cell Science Center. Properties of the α-PKC-specific monoclonal antibodies have been described (13). Rhodamine-phalloidin was from Molecular Probes (Seattle, WA). fluorescein-conjugated second antibodies were from Jackson Immunoresearch Laboratories (West Chester, PA). R-rabbit polyclonal anti-α-PKC antibody (St. Louis, MO).

Table 1 Effect of the pseudosubstrate peptide and anti-α antibodies on cell migration

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Goat IgG</th>
<th>M4</th>
<th>M9</th>
<th>Pseudosubstrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>13.0 ± 1.3</td>
<td>13.3 ± 0.4</td>
<td>13.5 ± 0.2</td>
<td>13.0 ± 0.9</td>
</tr>
<tr>
<td>12</td>
<td>8.8 ± 0.3</td>
<td>9.2 ± 0.9</td>
<td>10.1 ± 0.7</td>
<td>9.8 ± 0.9</td>
</tr>
<tr>
<td>24</td>
<td>4.4 ± 0.5</td>
<td>3.6 ± 0.8</td>
<td>5.0 ± 0.8</td>
<td>7.1 ± 0.3</td>
</tr>
<tr>
<td>36</td>
<td>1.8 ± 1.0</td>
<td>2.5 ± 1.0</td>
<td>2.5 ± 1.0</td>
<td>7.1 ± 1.0*</td>
</tr>
</tbody>
</table>

* P < 0.05.
Table 2  Effect of the pseudosubstrate peptide and the anti-α-PKC antibodies on cell attachment and growth

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>Control</th>
<th>Goat IgG</th>
<th>M4</th>
<th>M9</th>
<th>Pseudosubstrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>79 ± 7</td>
<td>77 ± 1</td>
<td>78 ± 2</td>
<td>77 ± 3</td>
<td>72 ± 7</td>
</tr>
<tr>
<td>24</td>
<td>153 ± 27</td>
<td>172 ± 28</td>
<td>160 ± 20</td>
<td>172 ± 22</td>
<td>117 ± 21*</td>
</tr>
<tr>
<td>48</td>
<td>343 ± 83</td>
<td>379 ± 64</td>
<td>365 ± 35</td>
<td>375 ± 53</td>
<td>274 ± 69*</td>
</tr>
</tbody>
</table>

* P < 0.05.

Grove, PA). HA-1004 was from LC Services (Woburn, MA).

**Cell Culture.** REF52 cells were obtained from D. McClure (Eli Lilly and Co., Indianapolis, IN). Cells were maintained and grown in a 3:1 mixture of Dulbecco's modified Eagle's and Ham's F12 media containing 10% fetal bovine serum.

**Phosphorylation in Permeabilized Cells.** The procedure described by Erusalimsky et al. (17) was used with some modifications. Subconfluent REF52 cells were washed twice with PBS and twice with an isotonic salt solution [120 mM potassium chloride, 30 mM sodium chloride, 1 mM magnesium chloride, 1 mM potassium phosphate, 10 mM sodium piperazine-N,N'-bis-(2-ethanesulfonic acid), 1 mM ethyleneglycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid, and 50 μM calcium chloride] at room temperature. Phosphorylation was performed by adding permeabilization buffer, i.e., isotonic salt solution containing 40 μM digitonin-10 μM [32P]ATP (1 Ci/mmol) in the absence or presence of 200 nM PDBu. The cells were incubated at 37°C for 5 min. The digitonin-soluble and -insoluble fractions were collected separately and boiled for 5 min. The heat-soluble proteins from both fractions were collected after centrifugation and concentrated by 10% trichloroacetic acid precipitation. Samples were solubilized in Laemmli sample preparation buffer (23). Proteins were resolved on 7.5% denaturing polyacrylamide gels. Phosphoproteins were detected by autoradiography.

**Incorporation of Monoclonal Antibodies into Cells by Osmotic Shock.** An osmotic shock procedure (24) was used to introduce antibodies into living cells. Subcon-
fluent REF52 cells were trypsinized and washed with a 3:1 mixture of Dulbecco’s modified Eagle’s and Ham’s F12 media containing 10% fetal bovine serum. The cells were incubated at 37°C in a hypotonic medium (50 µl/10⁶ cells) containing 0.9 M sucrose, 5 mg/ml antibody or 0.7 mg/ml pseudosubstrate peptide, and 10% polyethylene glycol 1000 in medium (pH 6.4) for 20 min. Monoclonal antibodies were purified from ascites fluid by Protein A-Sepharose chromatography. The pseudosubstrate peptide was purified by C18 reverse phase chromatography. The cells were diluted to 10 ml with pH 6.4 medium containing 10% fetal bovine serum and incubated for 2 min at 37°C. The medium was removed by centrifugation, and cells were washed twice with normal serum containing medium. The cells were plated and grown for 4–6 h for experiments.

α-PKC Redistribution After Wounding. REF52 cells were grown on coverslips to confluence and were wounded by scratching a line across the monolayers with a toothpick. At intervals, cells were fixed with 3.7% formaldehyde for 10 min and permeabilized with absolute acetone for 5 min at −20°C. Coverslips were stained with anti-α-PKC mAb M6. After washing, cells were stained with fluorescein-conjugated anti-mouse antibody and rhodamine-phalloidin as described (14, 22, 25).

Cell Migration after Wounding. Confluent REF52 cell monolayers were wounded by scraping the monolayers with a toothpick. Cultures were returned to the incubator for the indicated times and then fixed with formaldehyde and stained with 0.1% crystal violet. Migration was monitored by measuring wound widths on photomicrographs of duplicate samples at three points.

Cell Adhesion and Growth. Cells were plated into 16-mm wells at a density of 4 × 10⁴ cells/well. At intervals, cultures were rinsed to remove nonadherent cells. Attached cells were removed by trypsin treatment and counted in a Coulter counter. The original cell number plated onto the wells was set at 100% for calculations.

Incorporation of Monoclonal Antibodies into Confluent Monolayers by Scrape-Loading. REF52 cells were grown on coverslips to confluence. The cells were washed twice with PBS and were overlayed with 5 mg/ml of the various antibodies in PBS. The cells were scraped by scratching a line across the coverslips with a toothpick to introduce antibodies into cells bordering the artificial wound. At several time intervals, the cells were fixed with 3.7% formaldehyde in PBS for 10 min, permeabilized with absolute acetone for 5 min at −20°C, and stained with appropriate fluorescein-conjugated secondary antibodies. Fluorescence and phase contrast photomicrographs of four or five fields were taken.

References