

Association of Type 3 Protein Kinase C with Focal Contacts in Rat Embryo Fibroblasts

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Abstract. We have used immunocytofluorescence techniques to determine the subcellular distribution of the Ca^{2+} , phospholipid-dependent protein kinase, protein kinase C (PKC). Using monoclonal antibodies that are specific for Type 3 (α) PKC, we have determined that there are at least two pools of PKC in normal rat embryo fibroblasts (REF52 cells): diffuse cytoplasmic and fiber-associated. Extraction with chelators and detergent before fixing and staining removes the cytoplasmic PKC. The fiber-associated staining remains in these cytoskeleton preparations. The cytoskeleton

Type 3 PKC staining closely resembles that of the focal contact protein vinculin and colocalizes with another focal contact protein, talin. Cytochalasin, but not colchicine, coordinately disrupts the staining pattern of vinculin and PKC. Activation of PKC by treatment with phorbol esters causes depolymerization of microfilaments and reorganization of vinculin staining. We propose that Type 3 PKC is a modulatory component of the focal contact and has a primary role in regulation of the association of microfilament bundles with the plasma membrane.

PROTEIN kinase C (PKC)¹, the Ca^{2+} and phospholipid-dependent kinase is recognized to be a key regulatory enzyme. Several growth factors and hormones which are essential for normal cell growth and differentiated function regulate the activation state of PKC (26). This is due to their ability to stimulate phospholipase C activity, and consequently, to increase cellular diacylglycerol, an endogenous regulator of PKC. Tumor-promoting phorbol esters mimic diacylglycerol; they also bind and activate PKC (7, 25). Therefore, prolonged activation of PKC by phorbol esters, which is used in tumor promotion protocols, is thought to play a major role in tumorigenesis. These data demonstrate the importance of PKC both in normal cell function and in carcinogenesis.

These results, which underscore the key regulatory role of PKC, have led to efforts to identify substrates for PKC which are relevant to the observed biological responses. Identification of specific substrates has been complicated both by the demonstration that many proteins can serve as PKC substrates *in vitro* and by the lack of specific PKC inhibitors. Furthermore, because phorbol esters and PKC activation have been linked to a wide variety of biological responses, it has been difficult to distinguish between primary and secondary effects of PKC action (26).

PKC is now recognized to be a group of enzymes encoded by a family of genes (reviewed in reference 27). If the isozymes have different substrate specificities, then PKC heterogeneity may also complicate identification of PKC substrates. Although little data exist to directly compare isozyme

substrate specificities, evidence for differential ability to phosphorylate the EGF receptor has been presented (14). Subtle differences in cofactor requirements among the isozymes have also been reported (16, 31). However, it is not yet known if biochemical differences in cofactor requirements contribute to differential isozyme activation. Clearly, type-specific reagents, such as mAbs, are required to address questions regarding the role of individual isozymes in specific biological processes.

Using biochemical fractionation techniques, PKC is found in both the soluble and particulate fractions. Activation with growth factors or hormones causes redistribution to the particulate fraction (11, 19). Presumably, membrane association, which implies proximity with the activating phospholipids, is required for PKC activity (26). We have considered that the primary targets for phosphorylation by activated PKC would be concentrated in proximity to the membrane-associated PKC. The nature of the components of the particulate fraction with which PKC associates has not yet been described. We have, therefore, directed our efforts towards identifying the membrane association sites of PKC.

To accomplish this goal, we have developed highly specific mAbs to Type 3 PKC (21), the most widely expressed isozyme (18). We now report the use of these mAbs for immunocytochemical localization of Type 3 PKC. Our results suggest that there are several pools of Type 3 PKC in normal rat embryo fibroblasts (REF52 cells). A substantial fraction of Type 3 PKC is a component of the focal contact, the specialized area of the cytoskeleton (CSK) that mediates attachment of the microfilaments with the plasma membrane (6, 9). Furthermore, our results indicate that activation of PKC by treating cells with phorbol dibutyrate (PDBu),

1. *Abbreviations used in this paper:* CSK, cytoskeleton; PDBu, phorbol dibutyrate; PKC, protein kinase C.

causes disruption of microfilament organization. Thus, PKC appears to be spatially located to phosphorylate proteins that are important in regulating CSK structure.

Materials and Methods

Immunochemical Reagents

All anti-CSK antibodies were obtained from Sigma Chemical Co. (St. Louis, MO) except for antitubulin which was obtained from Southern Biotechnology Associates (Birmingham, AL). Rhodamine-conjugated phalloidin was purchased from Molecular Probes Inc. (Junction City, OR). Fluorescein- and rhodamine-conjugated second antibodies were from Cappel Laboratories (Malvern, PA). Alkaline phosphatase-conjugated antibodies and substrates were from Promega Biotec (Madison, WI). Rabbit antitubulin antibody was a gift of Dr. Keith Burridge (Chapel Hill, NC).

Other Reagents

Cytochalasin B, colchicine, and PDBu were all from Sigma Chemical Co. Triton X-100, Affi-gel 10, and protein assay reagents were from Bio-Rad Laboratories (Richmond, CA). All other reagents were of the highest quality available.

Cell Culture

Rat embryo fibroblasts (REF52 cells) were used between passage 10 and 20. Cells were grown in a 1:3 mixture of Ham's F-12 and DME containing 10% FBS. REF52 cells were originally obtained from Dr. D. B. McClure (Eli Lilly and Co., Indianapolis, IN).

Antibodies to PKC

The preparation and properties of the anti-Type 3 PKC mAb M6 used in these studies are described in reference 21. This mAb is specific for Type 3 PKC; it does not recognize Type 1 or 2 PKC. The epitope recognized is within the catalytic domain.

Preparation of CSKs

Cells grown on coverslips were washed twice in modified microtubule stabilization buffer without GTP and with glycerol instead of polyethyleneglycol as described by Osborn and Weber (28). Soluble proteins were extracted during a 5-min incubation in microtubule stabilization buffer containing 0.2% Triton X-100, 10 μ g/ml leupeptin, and 10 μ g/ml agrotinin. After washing twice in microtubule stabilization buffer, samples were fixed and stained as described for whole cells.

Immunofluorescence Protocol

Cells were grown on 12-mm glass coverslips. Cells or CSKs were washed twice with PBS, then fixed for 10 min at room temperature in 4% formaldehyde in PBS. After washing twice with PBS, cells were permeabilized for 5 min in PBS containing 0.2% Triton X-100 and finally washed twice with PBS. Staining was performed by incubating for 1 h in first antibody diluted in PBS containing 10 mg/ml BSA (PBA), washing, then incubating in rhodamine- or fluorescein-conjugated second antibody diluted in PBA (1:300) for 1 h. M6 was used at a 1:20 dilution. Rhodamine-conjugated phalloidin was used at 1:40 dilution in PBA. Coverslips were viewed on a Zeiss Axioplan microscope with a 63 \times objective. Photomicrographs were taken on Kodak T Max 400 film using 8–15-s exposure times for PKC stained samples.

Immunoblot Analysis

CSKs were prepared from cells grown in 35-mm dishes as described above. CSK protein, which remained on the dish, was collected by scraping into RIPA (50 mM Tris-Cl, pH 7.4, 0.15 M NaCl, 1 mM EDTA, 1% deoxycholate, 1% Triton X-100, and 0.1% SDS). Protein from both the soluble and CSK fractions was precipitated with chloroform and methanol as described by Wessel and Flügge (33). Samples were resuspended in 80 ml of Laemmli sample preparation buffer (20 containing 5% β -mercaptoethanol for SDS-PAGE and subsequent transfer to nitrocellulose. Equal aliquots (30 μ l) were applied to the gel. Immunoblots were blocked with 5% instant nonfat milk

in TBS (50 mM Tris-Cl containing 0.5 M NaCl), incubated in M6 diluted in TBA (TBS containing 1% BSA), and finally in alkaline phosphatase-conjugated goat anti-mouse IgG diluted in TBA. At least two washes in TBS were included between each incubation. Color was developed according to the manufacturer's procedures.

Preparation of PKC-absorbed Antibody Solution

PKC was purified as described (16) with the addition of a phenyl-Sepharose column. Approximately 5 mg of PKC isozyme mixture of >90% purity was coupled to Affi-gel 10 according to the manufacturer's instructions. Conditioned medium from the hybridoma culture was concentrated by ammonium sulfate, dialyzed against PBS, and applied to the PKC-affinity column equilibrated in PBS. The column flow through was collected. The starting material was diluted to adjust the protein concentration to that of the nonadsorbed column flow through. We verified that the column flow through was depleted of anti-PKC mAb relative to the starting material by dot blots with purified Type 3 PKC. Protein was measured by the method of Bradford (3).

Results

Type 3 PKC Immunofluorescence in Whole Cells and CSKs

Subconfluent REF52 cells were fixed, permeabilized, stained with the anti-catalytic domain, Type 3 PKC specific mAb M6 and finally, with fluorescein-conjugated second antibody (Fig. 1). Diffuse cytoplasmic as well as nuclear staining was apparent. Some of the extranuclear staining appeared to line up on fibers in an arrowhead pattern. The specificity of the staining was demonstrated in several ways. First, fluorescence was diminished if M6 was omitted. Some nuclear staining was observed in these minus M6 controls, however. Therefore, although nuclear staining appears brighter in samples incubated with M6, it is difficult for us to evaluate specific nuclear staining in these preparations. Second, fluorescence was greatly diminished if the M6 solution was first passed over a PKC affinity column. Third, immunoblots of cell lysates stained with M6 revealed only one major immunoreactive band of 82 kD which comigrated with purified brain Type 3 PKC (data not shown, but see Fig. 3).

The fiber-associated staining pattern suggested that some of the Type 3 PKC may be associated with CSK components. We, therefore, prepared CSKs by extracting soluble components with chelator and detergent. The CSKs were then fixed and stained as described for whole cells. The diffuse background fluorescence observed in whole cells is not apparent in the CSK preparations (Fig. 2). However, much of the fiber-associated staining remains.

We used immunoblots to identify the immunoreactive species associated with the CSKs (Fig. 3). Both the soluble and CSK fractions contained mainly 82-kD PKC as the major immunoreactive species. A higher molecular mass cross-reactive species was also found in the soluble fraction. Pretreatment of the cells with PDBu for 10 min before preparation of the CSKs did not substantially alter the distribution of Type 3 PKC between the soluble and CSK fractions.

Comparison of PKC Staining with CSK Protein Staining Patterns

To determine to what CSK compartment PKC is associated, we compared the PKC staining pattern to that of known CSK proteins using available antisera. Type 3 PKC staining was decidedly different from the microfilament staining observed with phalloidin, the intermediate filament staining

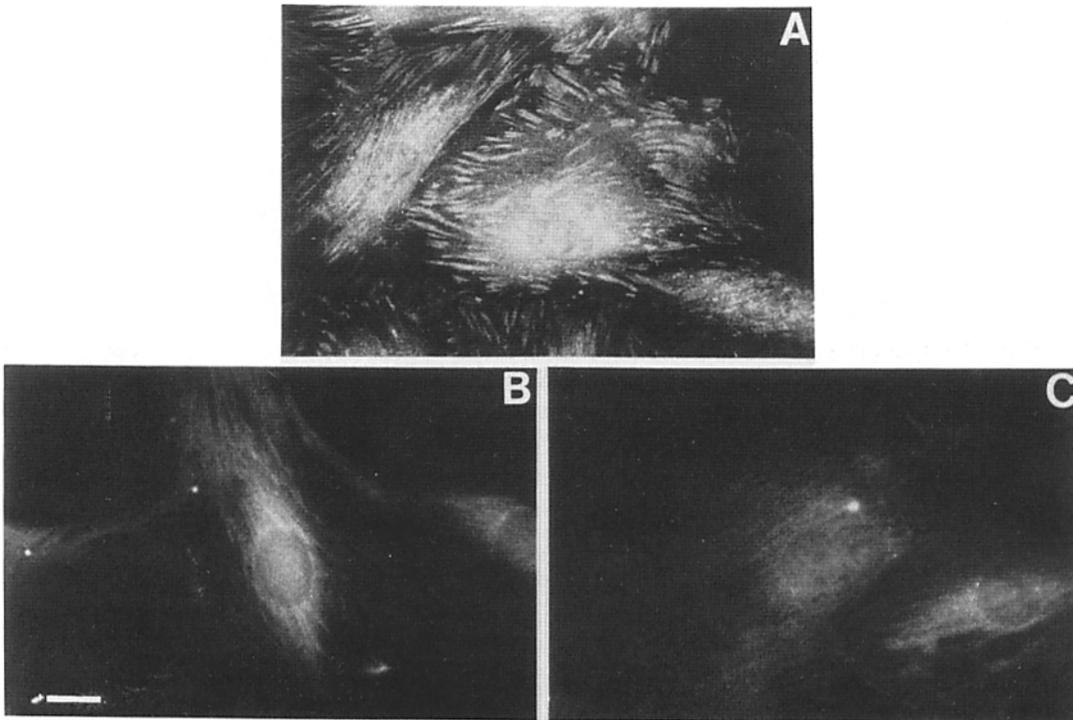


Figure 1. Type 3 PKC immunofluorescence in whole cells. REF52 cells (A-C) were fixed and stained as described in Materials and Methods. The first antibodies used were as follows: A, M6; B, minus first antibody control; C, PKC-absorbed M6 control.

observed with antivimentin, and the microtubule staining observed with antitubulin (Fig. 4). PKC staining was also different from that of the actin-binding proteins α -actinin, spectrin, filamin, and tropomyosin. However, Type 3 PKC

staining was very similar to that of the focal contact protein, vinculin. Double immunofluorescence with M6 and rabbit antibody to another focal contact protein, talin (5) demonstrates that staining patterns for Type 3 PKC and talin within the same cell are very similar (Fig. 5). In both cases, short strips of intense fluorescence were found throughout the cytoplasm. Nuclear staining is much more apparent with M6 than with vinculin or talin.

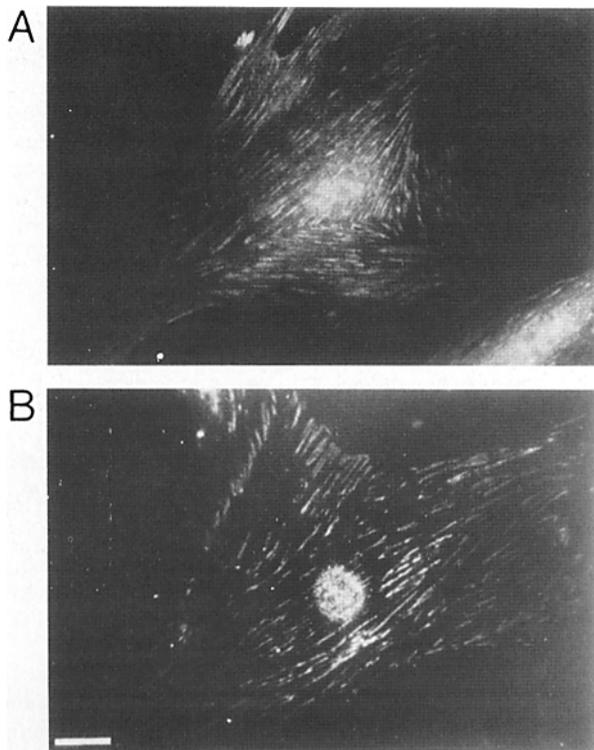


Figure 2. Type 3 PKC immunofluorescence in CSKs. Whole cells (A) or CSKs (B) were fixed and stained with M6.

The similarity in distribution of PKC with vinculin and talin indicates that PKC is also a component of the focal contact. In addition to its localization in the focal contact, talin has also been reported in ruffling membranes and at the leading edges of cells (5). It is therefore, possible that Type 3 PKC may also be found in other CSK structures. Some evidence for this can be seen in Fig. 5 (top) in which fibrils

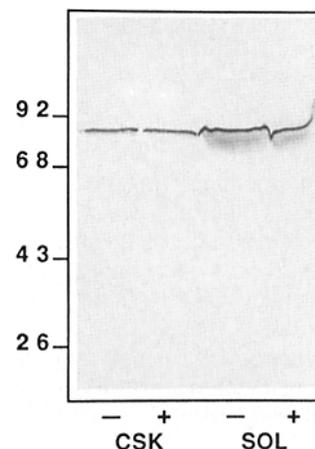


Figure 3. Immunoblot of REF52 cell fractions. Preparation of samples and immunoblotting protocol is described in Materials and Methods. Where indicated, cells were treated with PDBu (100 ng/ml) for 10 min at 37°. The soluble (SOL) and cytoskeletal (CSK) fractions from control (-) and PDBu-treated (+) cultures were analyzed with M6.

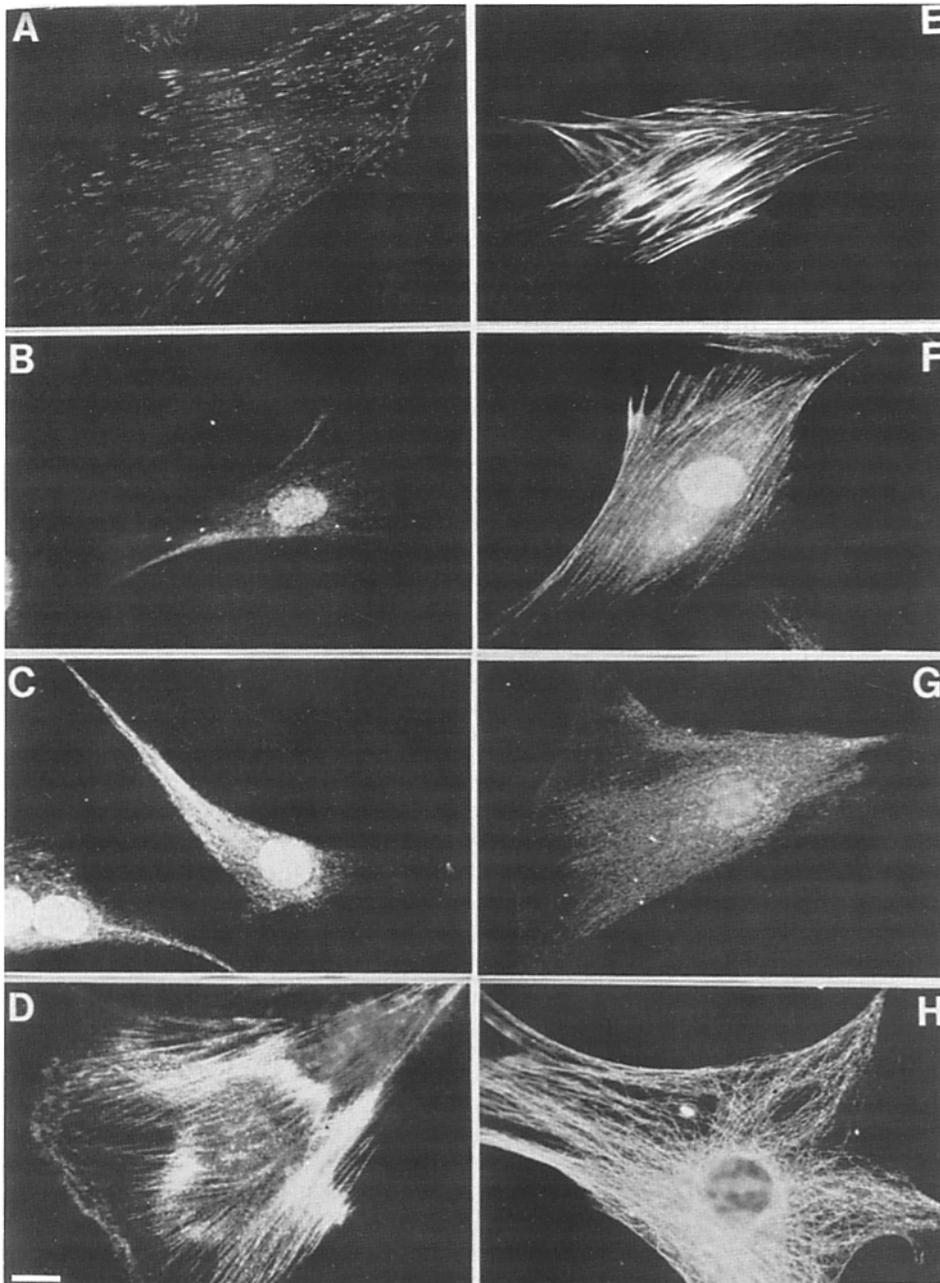


Figure 4. Immunofluorescence of CSK proteins in REF52 cells. CSKs were prepared, fixed, and stained as described in Materials and Methods. The first antibodies were directed against the following proteins: *A*, vinculin; *B*, α -actinin; *C*, spectrin; *D*, vimentin; *F*, tropomyosin; *G*, filamin; *H*, Tubulin. In *E*, the CSKs were stained with rhodamine-phalloidin.

staining for both PKC and talin appear to be above the nucleus, and therefore, cannot be at the substrate adhesion site.

Effects of CSK-altering Drugs on Type 3 PKC Staining

REF52 cells were treated with drugs that depolymerize specific CSK filaments to determine if PKC is directly associated with microfilaments or microtubules (Fig. 5). Colchicine caused depolymerization of microtubules without affecting the microfilaments (as observed with phalloidin staining). Colchicine did not significantly affect either PKC or vinculin staining. This demonstrates that PKC in the CSK is not associated with microtubules.

Treatment of REF52 cells with cytochalasin B caused marked disruption of microfilaments without disturbing microtubules (Fig. 6). Cytochalasin decreased the quantity and

size of the stress fibers observed with phalloidin. In addition, some completely depolymerized actin dots were found. Cytochalasin also changed vinculin and PKC staining patterns. In both cases, the fiber-associated staining was lost. Very short, thin, and disordered remnants of the fibers could be seen throughout the cytoplasm. An intense staining at the rim of the cells was also observed. The coordinate changes in vinculin and PKC staining with cytochalasin-treatment indicate that PKC and vinculin share a common subcellular localization.

Effects of Phorbol-Ester Treatment on Fluorescent Staining Patterns

The similar staining patterns observed for Type 3 PKC and the focal contact proteins, vinculin and talin, along with the

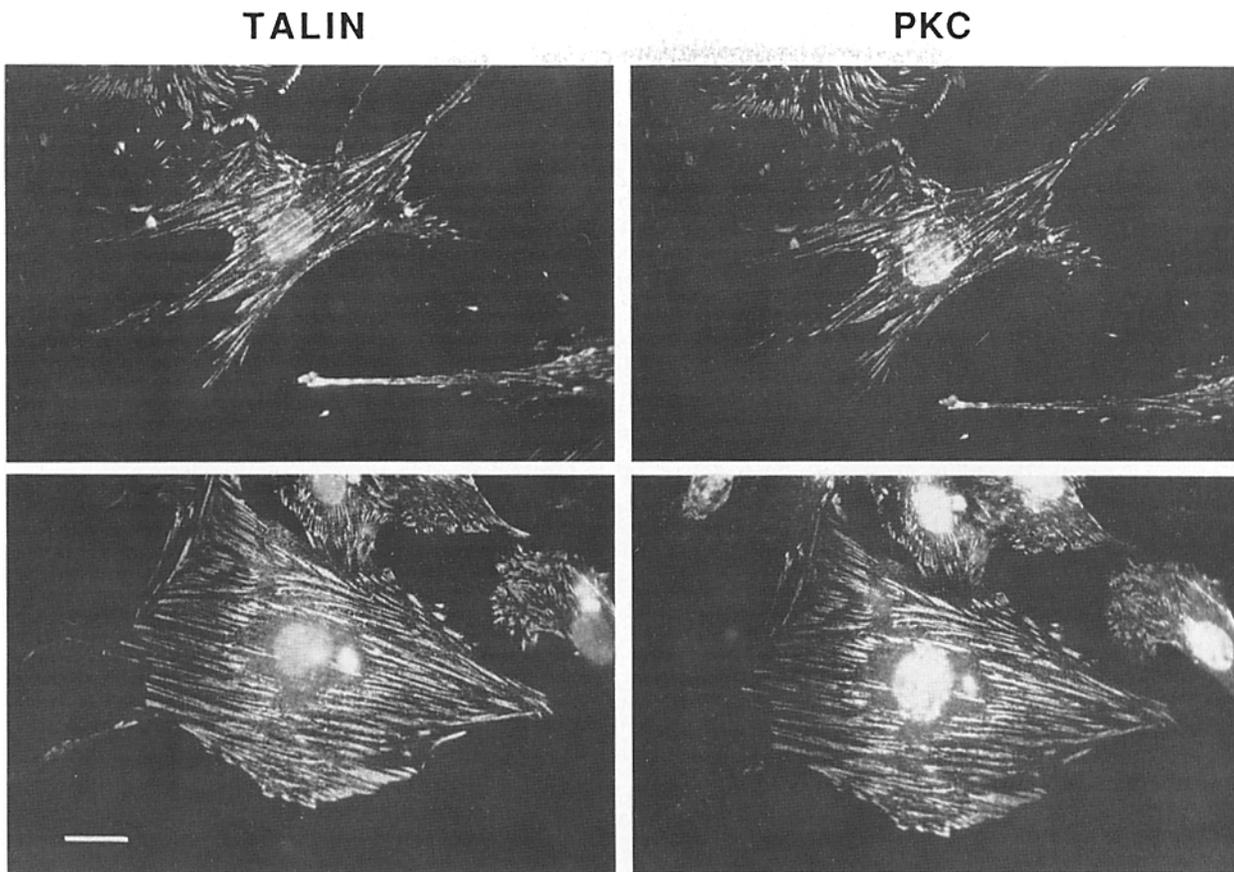


Figure 5. Colocalization of α -PKC and talin in REF52 cells. CSKs were prepared, fixed, and stained as described in Materials and Methods. Preparations were sequentially incubated with M6, rabbit antiserum to talin, and a mixture of TRITC-conjugated anti-rabbit and FITC-conjugated anti-mouse antibodies. Bar, 16 μ m.

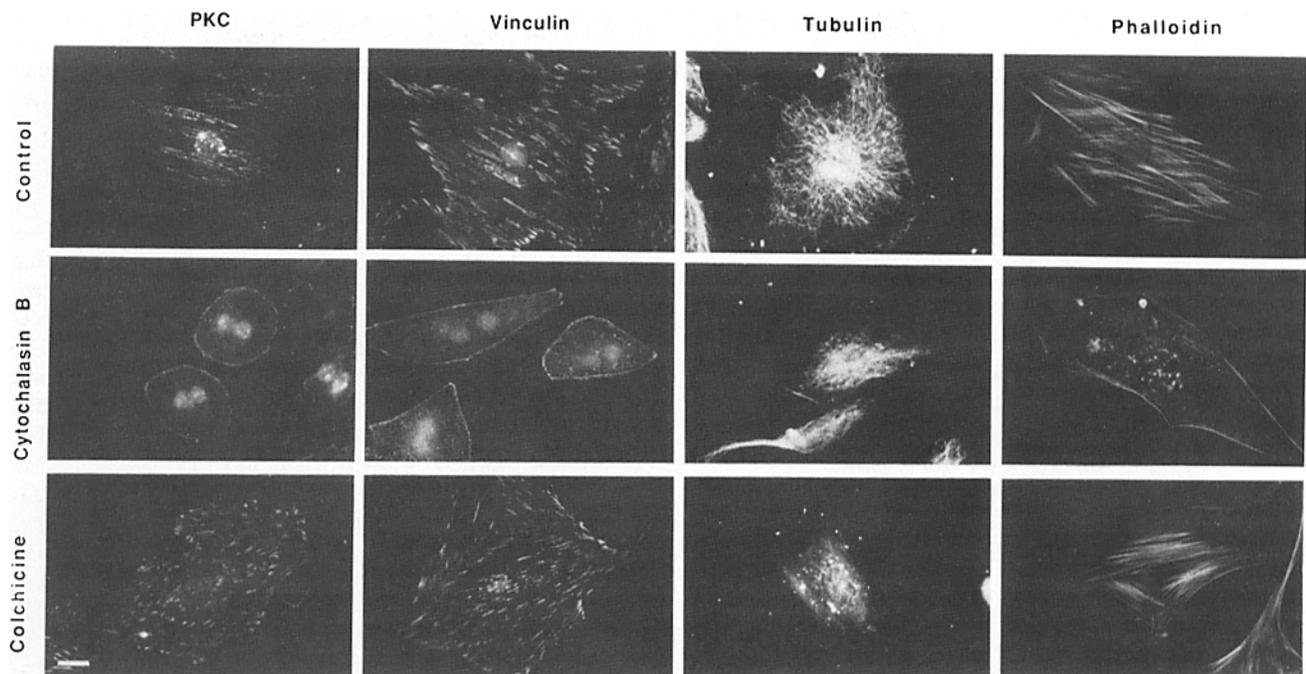


Figure 6. Effect of CSK-interacting drugs on PKC and vinculin staining patterns. Cells were treated with nothing (*control*), cytochalasin B (1 μ g/ml), or colchicine (1 μ g/ml) for 18 h. CSKs were then prepared, fixed, and stained as described in Materials and Methods.

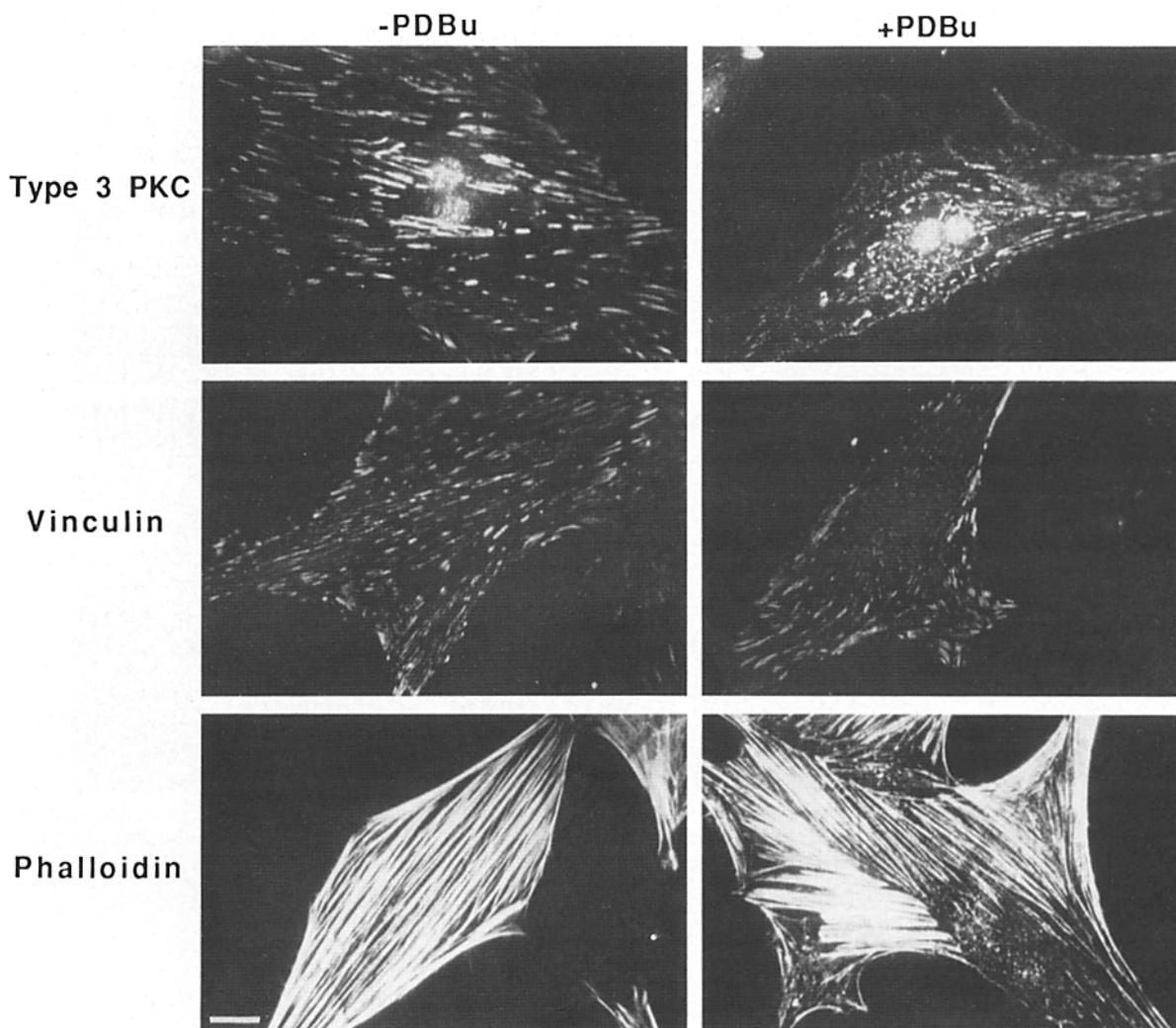


Figure 7. Effect of PDBu on PKC, vinculin, and microfilaments. Cells were treated without or with PDBu (100 ng/ml) for 10 min at 37°C. CSKs were prepared, fixed, and stained as described in Materials and Methods. Similar effects were observed in whole cell preparations.

cytochalasin experiments indicate that PKC is associated with focal contacts. Consequently, activation of PKC by treating cells with phorbol esters might lead to phosphorylation events that are important for regulating focal contact and/or microfilament structure. In fact, treatment of REF52 cells with PDBu causes a rapid depolymerization of microfilaments (Fig. 7). Actin bundles are less numerous and thinner. In some areas, complete depolymerization is seen. In PDBu-treated cells, vinculin staining in the cell interior is associated with shorter fibers and becomes disorganized. The staining no longer lines up in a parallel pattern. Effects on staining at the cell periphery are not as readily apparent. In PDBu-treated cells, changes in PKC staining paralleled those observed for vinculin.

Discussion

Cellular mechanisms for regulating the activation state of PKC are not clearly understood. In resting cells, PKC is found largely in the soluble fraction. Activation of PKC is

associated with redistribution to the insoluble fraction (19). The insoluble components with which PKC associates are undefined. Several lines of evidence suggest that interaction of PKC with the CSK is a likely candidate for regulating the partitioning of PKC between soluble and pelletable fractions. First, many CSK proteins are known to be good PKC substrates. These include several proteins associated with the actin cytoskeleton such as vinculin (32), filamin (17), profilin (12), and red cell band 4.1 (23). Phosphorylation of the intermediate filament protein, desmin, by PKC inhibits its ability to polymerize (15). Phosphorylation of the microtubule-associated protein MAP2 by PKC reduced its ability to interact with actin (1). PKC activation has also been associated with phosphorylation of the nuclear membrane protein, lamin B (8, 13). Second, phorbol esters cause morphological changes, which are likely a consequence of effects on CSK organization, in a variety of cells. Third, using biochemical techniques, Papadopoulos et al. demonstrated that PKC membrane association is chelator- and detergent-stable (27a). These are conditions similar to those we have used to prepare

CSKs from REF52 cells. Fourth, Wolf and Sahyoun found evidence that PKC may associate with detergent-insoluble CSK proteins using an overlay technique (34). We have extended these results using immunocytochemical techniques to demonstrate that the most widely expressed PKC isozyme, Type 3 (α), is in the CSK of REF52 cells. Furthermore, we were able to determine that a substantial portion of Type 3 PKC is concentrated in the specialized area of the CSK known as the focal contact.

Focal contacts are the specialized area of the cell that mediate attachment to the extracellular matrix (for reviews, see references 6 and 9). In this region, several proteins, including vinculin, talin, integrin, and α actinin mediate the attachment of microfilament bundles to the plasma membrane. Several less abundant proteins which may serve regulatory functions, have also been localized to this area, including calpain II (2) and the tyrosine kinases pp60src (29), p120 gag-abl (30), and p90 gag-yes (10). In addition, immunocytochemical studies using anti-phosphotyrosine antibodies indicate that tyrosine-phosphorylated proteins are also enriched in this area (24). Our data demonstrate that PKC Type 3 is also found in this region. Taken together, these data strongly suggest that the focal contact is not merely a structural link between the CSK, plasma membrane, and the extracellular matrix. Instead it appears to be a highly dynamic structure which functions in communication between the cell and the extracellular environment.

Vinculin, talin, and integrin play a functional role in attachment of microfilament bundles to the plasma membrane (6). There is evidence that each of these may be a PKC substrate (4, 6, 32). Our data demonstrate that one consequence of PKC activation in these cells involves reorganization of vinculin and depolymerization of actin. Our working hypothesis to explain these results is that PKC is a regulatory component of the focal contact. In resting cells, PKC is inactive. Upon stimulation with phorbol ester, PKC is activated and phosphorylates proteins that are important for stabilization of the microfilament-membrane interaction. This leads to depolymerization of the actin filaments, as well as deterioration of the adhesion plaque. This model allows us to postulate that proteins that mediate plasma membrane association of microfilaments are important PKC substrates, and allows us to design experimental approaches to determine which of these may be the primary targets for Type 3 PKC phosphorylation events. More importantly, these results provide a framework for understanding the multitude of phorbol ester-directed responses including changes in morphology, CSK organization, and adhesiveness (26).

Our data indicate that other pools of Type 3 PKC are also present in REF52 cells. In the accompanying article in which we have studied Type 3 PKC localization in 3T3 cells (22), several pools were also apparent including diffuse cytoplasmic, CSK, and nuclear. Because both studies used highly specific mAbs to Type 3 PKC, a direct comparison of the two cell types can be made. That is, our analysis is not complicated by the possibility that the mAbs are recognizing different PKC types in different cell lines. In 3T3 cells, the striking focal contact pattern observed in REF52 cells was not readily apparent. This difference may in part be due to the remarkably well-organized CSK characteristic of the REF52 cells. Consequently, the concentration of Type 3 PKC in the focal contact may be easier to discern in these cells compared with

others. The most notable feature of Type 3 PKC staining in the 3T3 cells was the redistribution to the nuclear envelope after phorbol ester treatment. This was not as readily observed in the REF52 cells. These differences indicate that Type 3 PKC has the potential for several different subcellular locations, and that individual cell type characteristics may influence the distribution. Such differences might stem from differences in the cellular Type 3 PKCs themselves, or to differences in the abundance of proteins with which Type 3 PKC associates. Further studies will address these issues.

We have demonstrated PKC localization in focal contacts of REF52 cells and the nuclear envelope of 3T3 cells (22). In spite of the specific differences, there is an underlying general theme in that Type 3 PKC is associated with CSK components in each cell line. More significantly, in each case, the association is with CSK structures that are of considerable importance in signal transduction and gene expression. In the REF52 cells, we demonstrated an effect of PKC activation by PDBu on focal contact structure. If we may extrapolate from focal contacts a similar function for PKC in other subcellular locations, we may suggest that Type 3 PKC may also function in regulating the association of other CSK filaments with other membranes, including the nuclear membrane. Higher resolution such as that obtained with the electron microscope will be used to address these issues.

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