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Type 3 Protein Kinase C Localization to the Nuclear Envelope of Phorbol Ester-treated NIH 3T3 Cells

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Abstract. We have examined the immunocytochemical localization of protein kinase C (PKC) in NIH 3T3 cells using mAbs that recognize Type 3 PKC. In control cells, the immunofluorescent staining was similar with mAbs directed to either the catalytic or the regulatory domain of PKC. Type 3 PKC localized in a diffuse cytoplasmic pattern, while the nuclei were apparently unstained. Cytoskeletal components also were stained. Treatment of the cells with phorbol 12-myristate 13-acetate (PMA) resulted in a redistribution of PKC with a specific increase in nuclear PKC. Compared to control cells, the staining with the anticatalytic domain mAbs changed markedly, covering the entire cell surface. In contrast, the staining by the antiregulatory domain mAb did not cover the cell surface and the nuclei remained unstained; these results suggest that PKC activation leads to a conformational change of the regulatory domain such that the epitope recognized by the antiregulatory domain mAb is not readily accessible.

We have demonstrated by three criteria that PMA treatment specifically increased PKC in the nucleus: (*a*) immunofluorescent staining in isolated nuclei increased; (*b*) Western blots showed that our mAbs detected only one protein, the 82-kD PKC, whose level increased in nuclear lysates from PMA-treated cells; and (*c*) PKC activity increased in nuclear lysates. In fractionation studies we demonstrated that PKC specifically localized to the nuclear envelope fraction. These results demonstrate that PMA activation leads to a rapid redistribution of Type 3 PKC to the nuclear envelope, and suggests that this isozyme may play a role in mediating PKC-induced changes in gene expression.

PROTEIN kinase C (PKC)[1] is involved in the regulation of many cellular processes, including differentiation, hormone and neurotransmitter release, and gene activation. Cloning studies have demonstrated that PKC is a family of closely related genes which encode a number of isozymes. Initially, three different PKC genes (α, β, γ) were found; additional cDNA clones (δ, ϵ, ζ) have subsequently been isolated. The α, β and γ gene products (also referred to as Types 3, 2, and 1 PKC, respectively) have been purified from a variety of different cells and tissues, and the δ, ϵ, and ζ products have been obtained from expression of the corresponding cDNA clones (for review, see reference 47).

The isoforms are highly homologous and all possess kinase and phorbol ester binding activities. The biochemical characterization of all the PKC isozymes is not complete, but some differences have been demonstrated. We and others (18, 22, 35, 38, 67) have isolated antibodies specific for the α, β, and γ isozymes, indicating that the proteins have unique antigenic determinants. The isozymes vary in their requirements for enzymatic activity as well. Jaken and Kiley (25) demonstrated that Type 1 PKC is less calcium dependent than Types 2 and 3. The Type 3 enzyme is more fully activated by arachidonic acid than are Types 1 and 2 PKC (59). Ido et al. (23) showed that the EGF receptor is phosphorylated most rapidly by Type 3 PKC in vitro suggesting that the different PKC species may have different substrate proteins. These results suggest that the different forms of PKC may mediate different cellular functions in vivo. However, the modes of activation within cells and the exact physiological substrates of the individual isozymes are not yet known.

1. *Abbreviations used in this paper*: PBA, PBS containing 1% BSA; PDBu, phorbol dibutyrate; PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate.

2. We were unable to precisely assess the percent of total PKC activity represented in the nuclear fraction after PMA treatment of the cells. We observed in seven experiments that the total picomoles of PKC activity recovered from the PMA-treated cells was only 50–70% of the total activity from the control cells. This was not due to incomplete extraction of PKC membrane activity from PMA-treated cells, since we verified by immunoblotting that our NP-40 extraction procedure removed all the immunoreactive Type 3 PKC from the membrane fractions (data not shown). PMA treatment did not alter the migration of PKC on the DEAE columns, since elution of the columns with increasing NaCl concentrations did not improve the recovery of PKC. Furthermore, immunoblots did not reveal the presence of PKC fragments. This indicates that the decrease in PKC is not due to proteolysis. Thus, the reason for the decreased recovery of PKC activity from PMA-treated cells is not readily apparent.

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Isozyme localization in different cell types or tissues may be a determinant of isozyme function, by regulating accessibility to substrates. Differential expression of isozymes within brain regions has been demonstrated (21, 56, 60). For example, in brain, Type 1 (γ) is highest in the cerebellum and hippocampus, while Type 2 (β) is highest in the cerebral cortex (60).

Substrate accessibility and thus isozyme function may also be regulated by isozyme localization to different subcellular compartments. PKC activity in control cells is primarily found in the 100,000-*g* supernatant fraction. Activation of PKC leads to a redistribution of enzyme activity to the membrane fraction (for review, see reference 46). There are a number of unanswered questions concerning PKC redistribution and the role of the individual PKC isozymes. First, it is not known whether redistribution is identical for all the isoforms. For example, differential sensitivity to activators such as diacylglycerols may regulate isozyme activation and redistribution. Second, the membrane compartment(s) to which PKC becomes associated have not been clearly identified by either biochemical or immunological analysis. For example, several studies have examined nuclear localization of PKC with varying results (15, 17, 34, 36). Some of the variability among these reports may be explained by the antibodies or the cell types used. In addition, differential localization of PKC isozymes may help explain some of the differences in these reports. To date, specific PKC isozyme localization to the cell nucleus has not been reported.

One approach to answering these and other questions concerning isozyme function is with type-specific mAbs. Such reagents allow the examination of the individual isozymes and their unique properties. We have developed highly specific mAbs to Type 3 PKC (38). In this and the accompanying article (26), we have used these mAbs to study the specific cellular localization of Type 3 PKC in cells. In this work, we examined the localization of Type 3 PKC in control and phorbol 12-myristate 13-acetate- (PMA) treated NIH 3T3 cells. Furthermore, using three different techniques, we have observed the phorbol ester–induced association of Type 3 PKC with nuclei. Nuclear subfractionation indicated that the bulk of the nuclear enzyme is associated with nuclear envelopes.

Materials and Methods

Materials

Antibodies and their sources were as follows: rat anti-tubulin (Sera-Lab, Westbury, NY); FITC-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Gaithersburg, MD); TRITC-labeled goat anti-rat IgG (Cappel Laboratories, Malvern, PA). PMA was from Sigma Chemical Co. (St. Louis, MO). Gelvatol was from Monsanto Co. (St. Louis, MO). The anti-PKC mAbs were as described in reference 38 and were from ammonium sulfate-precipitated hybridoma culture media.

Methods

Immunocytofluorescence. NIH 3T3 cells were plated on glass coverslips at 2×10^4 cells per coverslip and cultured in DME supplemented with 10% FCS. For experiments involving phorbol ester treatment, cells were treated with 200 nM PMA in 0.1% DMSO. Cells were fixed by dipping in −20°C acetone and washed three times in PBA (PBS containing 1% BSA and 0.2% sodium azide). Identical results were obtained with fixation in 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Fixed cells were incubated for 30 min at room temperature with anti-PKC mAbs diluted in PBA, washed three times with PBA, incubated in FITC- or TRITC-labeled second antibody for 30 min, and washed with PBA. For nuclear preparations, the nuclear pellet was washed once in 10 mM Tris, pH 7.5, containing 10 mM NaCl, 10% sucrose, and 1 mM $MgCl_2$, and resuspended in PBA and cytospun at 250 rpm for 5 min onto glass slides. The nuclei were fixed by dipping in −20°C methanol and stained as described for the intact cells. The coverslips were mounted on slides with Gelvatol. Fluorescence microscopy was carried out with a Zeiss Photomicroscope II using a 25× objective. Photo micrographs were made using Kodak Tri-X Pan film.

Cytoskeleton Preparations. Cytoskeletons were prepared using 100 mM Pipes buffer, pH 6.9, containing 0.2% Triton X-100, 1 mM EGTA, 2.5 mM GTP, and 4% polyethylene glycol 8,000, as described in references 49 and 50. The cytoskeletons were fixed in 1% paraformaldehyde for 10 min and stained as described for whole cells. In experiments not shown, cytoskeletons from control and PMA-treated cells were prepared in Pipes buffer containing 0.05, 0.1, 0.2, and 0.5% Triton X-100. In all cases no differences were observed in nuclear staining in the cytoskeletons from PMA-treated cells, as compared to the control. The same experiment was also carried out using the modified Pipes cytoskeleton buffer described by Jaken et al. (26). No change in nuclear staining in cytoskeletons from PMA-treated cells was observed under these conditions as well.

Subcellular Fractionation. 3T3 cells grown on 150-mm plates were treated with either 0.1% DMSO (control) or 200 nM PMA for 10 min at 37°C. The cells were washed twice with saline and incubated for 30 min on ice with saline containing 1 mM EDTA. Cells were removed from the plates using a Pasteur pipette, centrifuged at 500 *g* and washed twice with saline. The cell pellet was resuspended in nuclei buffer (10 mM Tris, pH 7.5, 10 mM NaCl) containing 0.1% Triton X-100, homogenized 10× in a Dounce homogenizer with a tight-fitting pestle and spun at 500 *g* for 5 min. The resulting supernatant, determined to be free of nuclei as judged by light phase microscopy, was centrifuged at 100,000 *g* for 60 min. The 100,000-*g* pellet was resuspended in DEAE column buffer (20 mM Tris, pH 7.5, 2 mM EDTA, 0.5 mM EGTA, 5 mM DTT) containing 1% NP-40 and rocked for 1 h at 4°C. Neither this pellet nor the 100,000-*g* supernatant contained lamin B, as determined by Western blots. The 500-*g* nuclear pellet was resuspended in nuclei buffer and layered over 45% sucrose in nuclei buffer and centrifuged at 1,660 *g* for 30 min. The nuclear pellet was washed twice in nuclei buffer containing 10% sucrose and 1 mM $MgCl_2$, and resuspended in DEAE buffer for Western blots, 1 mM $KHCO_3$ for adenylate cyclase assays, or in DEAE buffer containing 1% NP-40 for PKC assays. In experiments not shown, we established that 1% NP-40 (or Triton X-100) was required to extract PKC activity from nuclear and nonnuclear membrane fractions. Treatment with either 0.1 or 0.2% detergent extracted no PKC activity from these fractions, and 0.5% detergent extracted only 30% of the activity extracted by 1% detergent.

In our initial experiments, the cells were homogenized in the complete absence of Triton X-100. Using this method, it was necessary to homogenize the cells 45–55× in a Dounce homogenizer with a tight-fitting pestle. We were able to demonstrate PMA-induced PKC nuclear localization under these conditions; however, we found that our yield of nuclei was quite low. We, therefore, included 0.1% Triton X-100 in the homogenization buffer, which allowed us to decrease the homogenization pestle strokes and to increase our nuclei recovery to >90%.

Nuclear Envelope Preparations. Nuclear envelopes were prepared as described in reference 33. Briefly, nuclei were resuspended in STM buffer (250 mM sucrose, 50 mM Tris, pH 7.5, 5 mM $MgSO_4$), incubated with RNase and DNase, followed by extraction in 1.6 M NaCl and 1% β-mercaptoethanol (vol/vol).

PKC Assays. The nuclear and 100,000-*g* membrane detergent extracts, and the 100,000-*g* supernatants were applied to 1.0 ml DEAE columns, washed with DEAE buffer, and eluted with DEAE buffer containing 120 mM NaCl. Samples were assayed for kinase activity either in the presence of 0.8 mM $CaCl_2$ and 200 nM PDBu, or in the presence of $CaCl_2$, PDBu, and 100 μg/ml phosphatidylserine, as described in reference 38.

Immunoblots. Western blots were carried out as previously described (38). Blots were erased using the procedure of Kaufmann et al. (33).

Adenylate Cyclase. Adenylate cyclase was assayed as described by Salomon (57) on fractions from both control and PMA-treated cells, in the absence of detergents.

Superoxide Dismutase. Superoxide dismutase (1) was assayed in 96-well plates containing, in a total volume of 250 μl, 50 mM Tris, pH 7.8, 20 mM EDTA, 2.5 μg/ml riboflavin, 75 μg/ml nitro blue tetrazolium, and 0–100 μg of each of the subcellular fractions. The plates were incubated for 10 min at room temperature on a fluorescent light box, and color development was measured on a Titertek multiscan plate reader with a 600-nm filter.

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Nuclear Protein Antibodies. Rat liver nuclear envelopes (31) or internal nuclear matrix polypeptides (30) were subjected to two-dimensional IEF/SDS-PAGE. Individual polypeptides were excised and injected into chickens or guinea pigs according to the schedule previously described (12). Antibodies against the nuclear envelope lamins and the 38-kD nucleolar polypeptide B23 have been previously described (12). The antibody against the 62-kD nuclear matrix/chromosomal scaffold polypeptide is described by Newitt, J., J. Shaper, and S. Kaufmann (manuscript in preparation).

Results

Cellular Immunofluorescence

In an earlier study (38), we reported on the characterization of several mAbs to Type 3 PKC: M4, a mAb that recognizes the catalytic domain of Type 3 PKC; M7, an anticatalytic domain mAb that recognizes an epitope common to both Types 2 and 3 PKC; and M9, a mAb directed against the regulatory domain of Type 3 PKC. In this study we have used these mAbs to follow the immunocytochemical localization of PKC in NIH 3T3 cells; McCaffrey et al. (43) reported that NIH 3T3 cells contain only α (Type 3) PKC. The cells were fixed, permeabilized, and stained with the anticatalytic Type 3 PKC mAb M4 (Fig. 1). In control cells, the staining was diffuse and localized to the cytoplasm. The nuclei were visible due to the absence of staining (Fig. 1 *A*). Treatment of the cells with 200 nM PMA for 10 min markedly changed the staining pattern (Fig. 1 *B*). The M4 immunofluorescence covered the entire surface of the cells, and the nuclei were no longer apparent. The staining pattern of cells treated with 4α phorbol 12,13-didecanoate, a biologically inactive tumor promoter, was identical to that of control cells (data not shown). On Western blots, M4 recognized the 82-kD PKC from both control and PMA-treated cells (see Fig. 5 *A*).

Similar staining under each of the cell treatments described (control or 10 min PMA) was observed with M6 (data not shown) and M7, two other anticatalytic domain PKC mAbs (Fig. 1, *C* and *D*). After a 10-min PMA treatment, the M7 staining covered the cell surface (Fig. 1 *D*). The antiregulatory domain mAb M9 stained control cells in a pattern similar to that of M4 and M7 (Fig. 1 *E*). The M9 immunoreactivity was localized to the cytoplasm, but the staining was more fibrous in appearance than that obtained with M4. After a 10-min PMA treatment, a decrease in the uniform cytoplasmic immunofluorescence was seen as compared to control (Fig. 1 *F*). In contrast to the anticatalytic domain mAb staining pattern, the antiregulatory domain M9 staining after PMA treatment did not cover the cell surface, and the nuclei remained unstained.

We verified that the staining by the PKC mAbs was specific using competition experiments (Fig. 2). Preincubation of M4 with rat brain PKC decreased the immunofluorescent staining in the cells. In ELISA assays, we showed that this preincubation of the mAbs also eliminated their reactivity with PKC purified from rat brain (data not shown).

Cytoskeletal Immunofluorescence

As with the REF52 cells (26), we examined immunofluorescent staining in 3T3 cytoskeletal preparations. Immunoblotting experiments demonstrated that the mAbs reacted only with the 82-kD PKC protein in these detergent, EGTA-extracted preparations (data not shown). The cytoskeletal extracts from control cells were double-stained with either M4 or M9 and a rat antitubulin antibody (Fig. 3). Incubation with the tubulin antibody resulted in bright microtubule staining (Fig. 3, *B* and *D*), and verified the cytoskeletal extraction procedure. Actin microfilaments also were present, as shown by phalloidin staining (data not shown). Both M4 and M9 stained these cytoskeletal preparations, although greater staining was observed with M9. For both mAbs, the staining patterns appeared fibrous, and the nucleus stained lightly. The staining pattern in cytoskeletal preparations from PMA-treated cells was not significantly different from the control cell preparations.

Nuclear Localization

Using the biochemical techniques of Western blots and kinase assays, we observed in our preliminary experiments that PMA treatment caused an increase in PKC activity and immunoreactivity in the 100,000-*g* crude particulate fraction (which includes nuclei) of the cells (data not shown). The M4 and M7 immunofluorescent staining patterns of PMA-treated cells covered the entire cell surface and suggested that the localization of PKC may include the nucleus. We, therefore, decided to examine nuclear localization of PKC in more detail, by fractionating control and PMA-treated cells into nuclear, 100,000-*g* supernatant and 100,000-*g* nonnuclear membrane fractions. The purity of the nuclei preparations was determined by adenylate cyclase and superoxide dismutase (SOD) assays, as well as by electron microscopy. The level of adenylate cyclase, a plasma membrane enzyme, in the nuclear lysates was <5% of that seen in the 100,000-*g* pellet fraction. Cytosolic contamination was measured by superoxide dismutase activity. Essentially no superoxide dismutase activity was measured in our nuclear fractions. Electron microscopic examination showed that the preparations contained intact nuclei, with some nuclei (~20%, based on visual inspection) having broken nuclear membranes (Fig. 4 *A*). Furthermore, the nuclei were relatively free of contaminating organelles.

PKC localization to the nuclear fraction was assessed in three ways: immunofluorescence on isolated nuclei, direct assay for enzyme activity, and Western blotting of isolated nuclei. First, in immunofluorescence experiments, nuclei isolated from control cells showed little staining with M4 (Fig. 4 *B*). In contrast, PMA treatment of the cells increased significantly the immunoreactivity of M4 in the nuclei (Fig. 4 *C*). The staining pattern covered the surface of the nuclei in a punctated manner and the intensity was somewhat heterogeneous among the nuclei.

Second, PKC activity was assayed in nuclear lysates. In preliminary experiments, we established that recovery of PKC activity from the nuclear lysates required detergent extraction (data not shown). Purified nuclei were extracted with 1% NP-40, and PKC activity in the nuclear extracts was measured after DEAE chromatography. Table I shows that in control cells, the specific activity of the nuclear fraction was low, 380 pmol/min per mg, and nuclear activity accounted for only 1–4% of the total cellular PKC activity. After PMA treatment, however, the specific activity in the nuclear fraction increased 10–20-fold. This activity was absolutely dependent on the presence of calcium and phospholipid.[2] The activity of the 100,000-*g* (excluding nuclei) pellet also increased with a concomitant decrease in the 100,000-*g* supernatant fraction, as expected.

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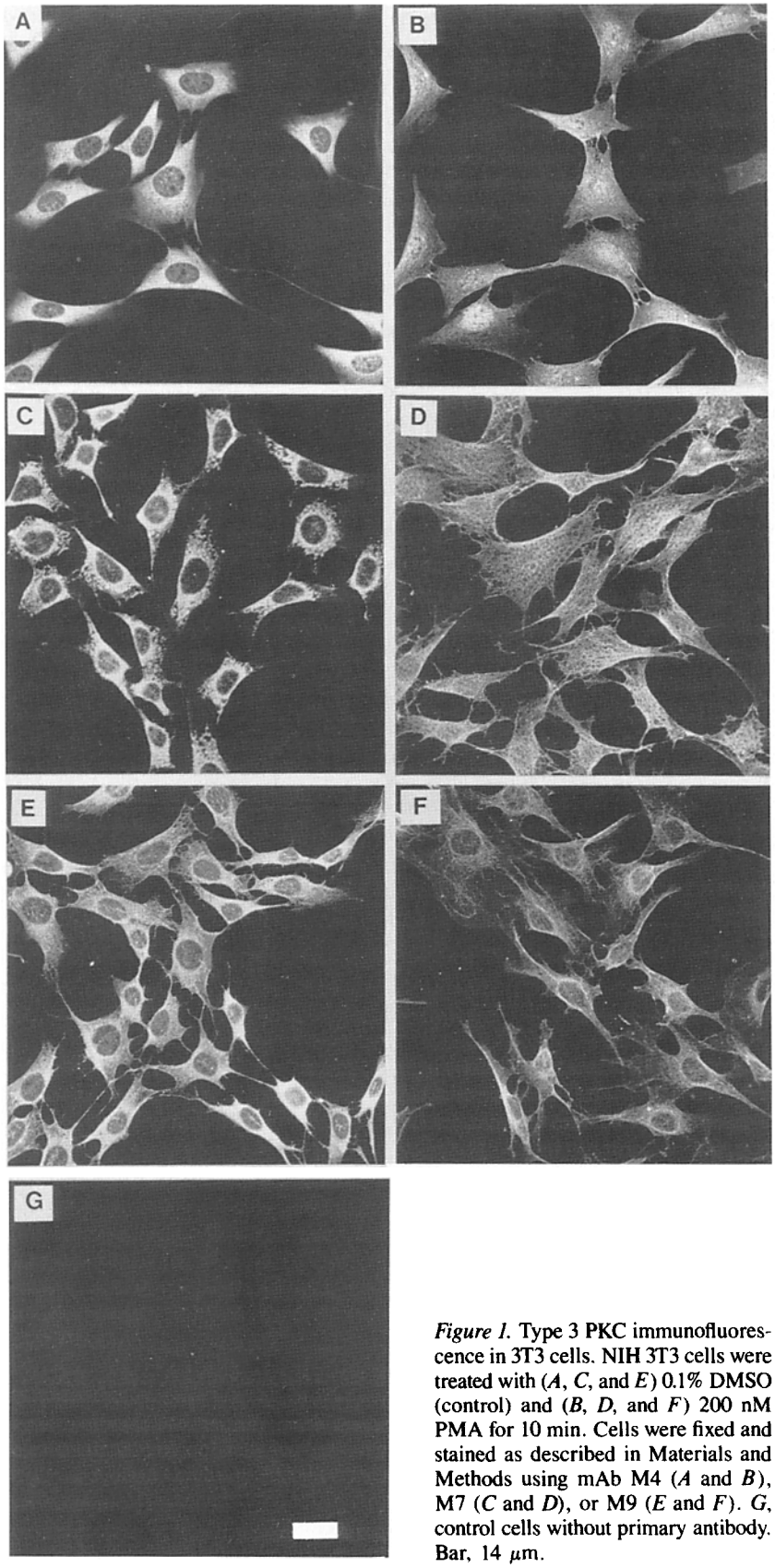

Figure 1. Type 3 PKC immunofluorescence in 3T3 cells. NIH 3T3 cells were treated with (*A*, *C*, and *E*) 0.1% DMSO (control) and (*B*, *D*, and *F*) 200 nM PMA for 10 min. Cells were fixed and stained as described in Materials and Methods using mAb M4 (*A* and *B*), M7 (*C* and *D*), or M9 (*E* and *F*). *G*, control cells without primary antibody. Bar, 14 μm.

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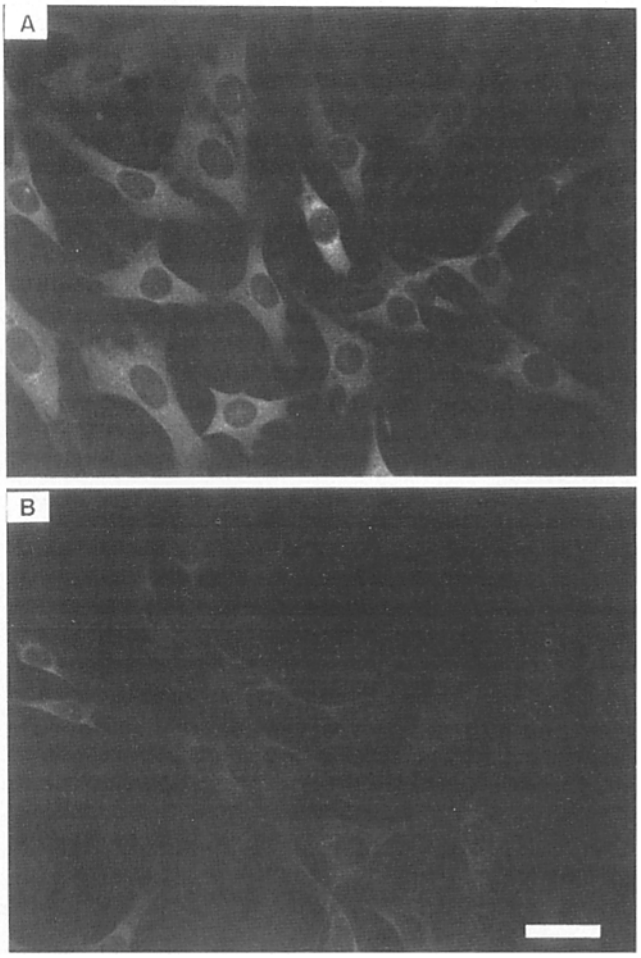

Figure 2. Competition of Type 3 PKC immunofluorescence by rat brain PKC. M4 was preincubated with PBA (*A*) or PKC partially purified from rat brain (*B*) for 30 min at room temperature. Staining of fixed cells was then carried out as described in Materials and Methods. Bar, 20 μm.

Third, the presence of PKC in the nucleus was assessed in Western blots (Fig. 5). In all the cell fractions, including the nuclear preparations, M4 recognized only one protein, which co-migrated with the 82-kD PKC purified from rat brain. M7 gave identical results to M4 on Western blots (data not shown). Type 3 PKC levels increased in the 100,000-*g* (nonnuclear) pellet of PMA-treated cells, with a concomitant decrease in the 100,000-*g* supernatant immunoreactivity (Fig. 5 *B*). Nuclear lysates from control cells contained little of the 82-kD immunoreactivity (Fig. 5 *C*, lane 1), which was consistent with our biochemical and immunofluorescent results. However, the amount of 82-kD PKC increased in the nuclei prepared from PMA-treated cells (Fig. 5 *C*, lane *2*). Densitometric scanning of the autoradiograms indicated that PMA treatment increased the level of Type 3 PKC ~30-fold in the nuclear fraction.

Using either M4 or M7, the presence of the 45-kD catalytic domain of PKC, which is recognized by both these mAbs, was not detected. This was true for all the subcellular fractions from both control and PMA-treated cells. In Western blots using M9, the presence of 82-kD PKC was not detected. This result was not surprising, since our earlier studies demonstrated that the antiregulatory domain mAb M9 reacts poorly with denatured 82-kD PKC on Western blots. However, we also did not observe the presence of the 35-kD regulatory domain of PKC, which is recognized by M9 on Western blots (38). Overall, these results demonstrate the presence of the 82-kD holoenzyme, but not PKC fragments, in the cellular fractions, including the nuclei, of control and PMA-treated cells.

To examine further the nuclear localization of PKC, we fractionated nuclei from untreated and PMA-treated cells as described by Kaufmann (31). This method separates intranuclear proteins (including the nucleoli), DNA and RNA, from nuclear envelopes. When these fractions were probed with M4, only the nuclear envelope fraction of the PMA-treated cells contained the 82-kD PKC (Fig. 5 *C*, lane *8*). When nuclear PKC in control cells was detected, it was always found in the nuclear envelope fraction as well. We did not detect the presence of PKC in either the RNase/DNase fractions (Fig. 5 *C*, lane *3* and *4*) or high salt extracts (Fig. 5 *C*, lanes *5* and *6*). We confirmed the effectiveness of the nuclear separation by probing with an antibody to lamin B, a 67-kD nuclear envelope protein (Fig. 5 *D*). The lamin B polyclonal antibody recognized a 67-kD protein in the nuclear lysates and the envelope fractions, but not in the intranuclear protein fractions. These results demonstrate that Type 3 PKC cofractionates with lamin B, a nuclear envelope polypeptide. We also probed the blots with antibodies to a 38-kD nucleolar structural protein and to a 62-kD intranuclear matrix protein. These proteins were present in the high salt nuclear extracts and nuclear lysates (data not shown). These experiments confirmed the efficiency of our fractionation protocol and demonstrated that cofractionation of PKC with nuclear envelope proteins was not due simply to incomplete separation of the nuclear fractions.

Discussion

The role of each of the PKC isozymes in cellular transduction pathways is not known. These proteins are highly homologous in their amino acid sequence, yet differences in their immunological and biochemical properties exist and may be important determinants of isozyme function. These properties include substrate specificity, cofactor requirements, as well as tissue distribution and subcellular location. Experiments to determine the role of the individual isozymes have been complicated by the lack of type-specific assays, reagents, and inhibitors. To study the role of an individual isozyme, we have prepared mAbs that are specific for Type 3 PKC and have used them to study the subcellular localization of this isozyme. We observed cytosolic and cytoskeletal staining in control cells, and we clearly demonstrated that PMA treatment causes a redistribution of PKC to the nucleus. This result has important implications in understanding the role of PKC in signal transduction and regulation of specific gene expression.

In control NIH 3T3 cells, PKC localization detected by mAbs directed towards either the catalytic or regulatory domains of PKC was similar. A diffuse, cytoplasmic staining was observed with little or no nuclear staining. Recently, Ito et al. (24) demonstrated that Type 2 PKC mAbs stain untreated human leukemia cells in a diffuse cytosolic pattern.

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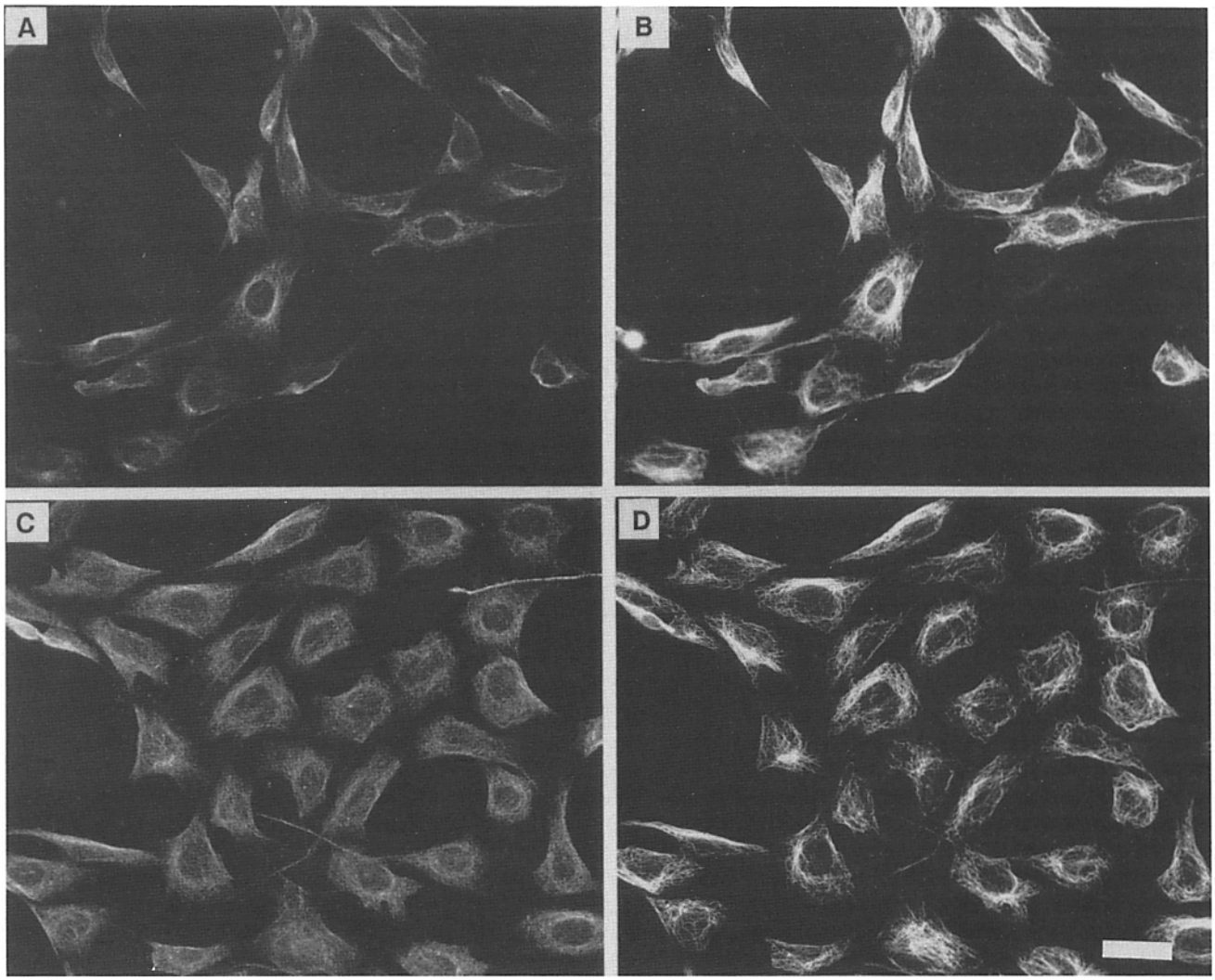

Figure 3. Immunofluorescence of 3T3 microtubule cytoskeleton preparations. Cytoskeletons were prepared, fixed, and incubated with rat anti-tubulin and mouse anti-PKC mAbs (M4 or M9) followed by TRITC-labeled goat anti-rat IgG and FITC-labeled goat anti-mouse IgG. Cytoskeletons were double stained with (*A* and *C*) M4 and rat anti-tubulin and (*B* and *D*) M9 and rat anti-tubulin. The PKC staining was viewed under green light (fluorescein) and the tubulin staining was viewed under blue light (rhodamine). PKC staining is shown in *A* (M4) and *C* (M9) and the tubulin staining is shown in *B* and *D*. Bar, 20 μm.

To our knowledge, this is the first report of the immunofluorescent localization of PKC with mAbs in intact NIH 3T3 cells.

Staining by M4 and M9 in detergent, chelator-treated cells indicated that at least part of the whole cell staining may be due to association of PKC with components of the cytoskeleton, since actin and intermediate filaments, as well as microtubules, were present in our cytoskeletal preparations. Our results differ from those of Thomas et al. (62), who found little or no staining in the cytoskeletal fraction of untreated NIH 3T3 cells. Differences in antibodies and the epitopes that they recognize may account for the differences between these studies. We found no significant increase in cytoskeletal staining after PMA treatment of the cells. Our results from preparing cytoskeletons with several different buffers (see Materials and Methods) suggested that immunofluorescent staining of the cytoskeleton is not a suitable method to study PKC localization in the nucleus. Therefore, we approached this question of nuclear localization more directly in studies using purified nuclei. In other studies, the association of PKC with cytoskeletal proteins has been reported (64, 65), and PKC has been shown to phosphorylate several cytoskeletal proteins, including talin (40), vinculin (63), and the microtubule-associated protein tau (20). We have demonstrated cytoskeleton-associated Type 3 PKC staining in NIH 3T3 cells, but further characterization is required to identify the specific cytoskeletal components which are involved. The cytoskeleton-associated Type 3 PKC in REF52 cells is examined in more detail in the accompanying manuscript (26).

Several studies have shown redistribution, by biochemical or immunocytochemical analysis, of PKC after PMA activation (for review, see reference 46). After a 10-min PMA treatment, PKC redistribution was observed in NIH 3T3 cells by immunostaining with the anticatalytic domain mAbs, M4 and M7. The staining pattern with both these mAbs covered the cell surface. This apparent PKC redistribution was not noted with the antiregulatory domain mAb M9. The M9 staining was clearly different in several ways.

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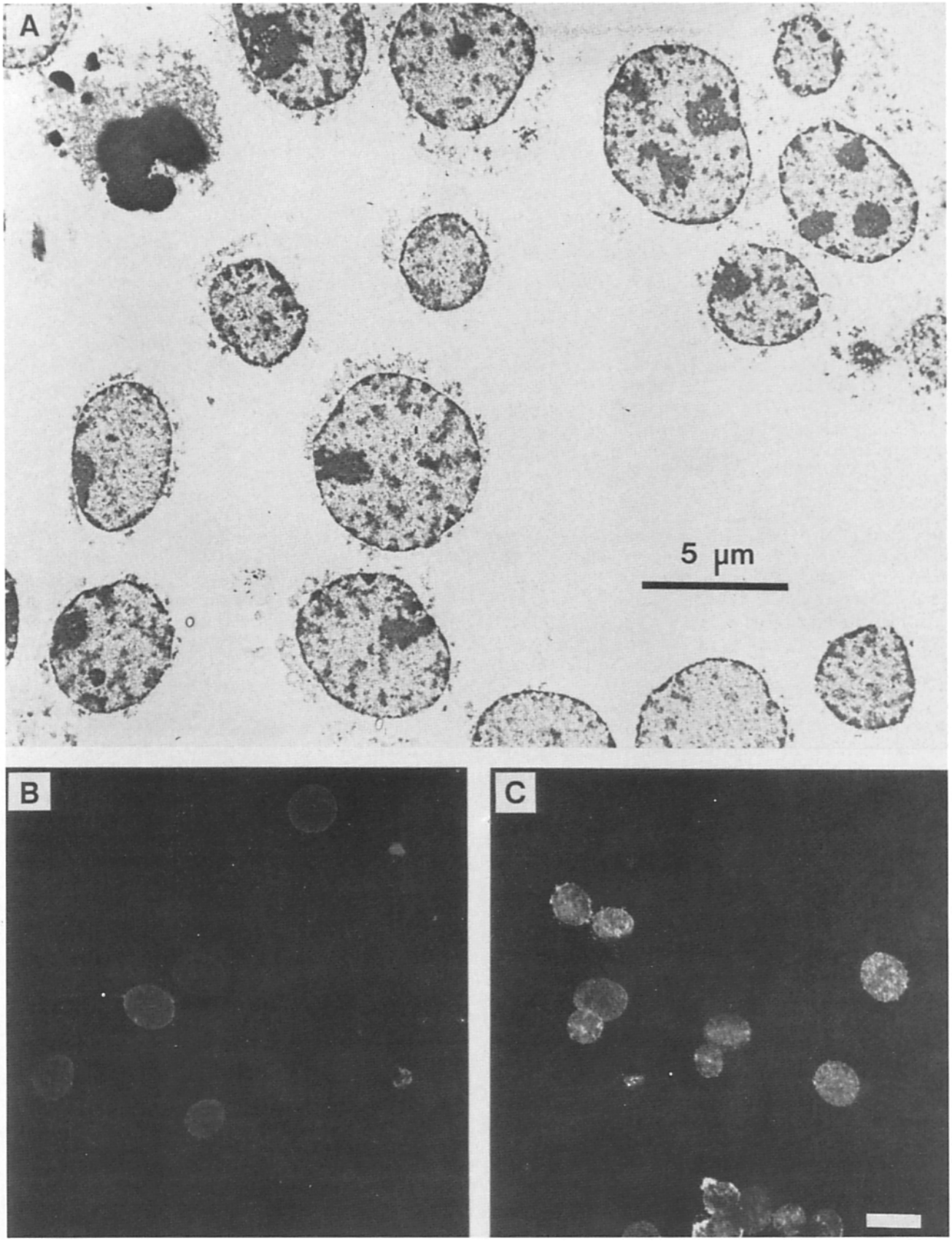

Figure 4. Isolated nuclei and Type 3 PKC immunofluorescence. (*A*) Electron micrograph of isolated 3T3 nuclei. Nuclei were isolated from control (*B*) or PMA-treated (10 min) (*C*) cells, fixed and stained with M4. Bar, 10 μm.

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Table I. PKC Activity in 3T3 Subcellular Fractions

	Control	PMA
	pmol/min per mg	
100,000 *g* supernatant	1973 ± 290	110 ± 28
100,000 *g* nonnuclear membrane pellet	1478 ± 392	5960 ± 122
Nuclear lysate	383 ± 188	7021 ± 2250

Cells were incubated in the presence or absence of 200 nM PMA for 20 min at 37°C and subcellular fractions and kinase determinations were carried out as described in Materials and Methods. The results are the average ± SEM of four experiments.

First, the nuclei remained unstained and the staining did not cover the entire cell surface. Second, the M9 staining was less uniform throughout the cytoplasm, with an increase in perinuclear staining. Western blot analysis of the PMA-treated cells (as well as control cells) showed only the 82-kD PKC (Fig. 5 *A*). The presence of either the 45-kD catalytic or the 35-kD regulatory domains, which are detected by M4 and M9, respectively, was not observed. This result suggests that the difference in PKC localization indicated by the staining with M4 and M9 is not due to differential localization of PKC fragments.

The different staining patterns observed with the anticatalytic domain mAbs M4 and M7 as compared to the antiregulatory domain mAb M9 may in part be explained by differences in their PKC recognition properties. Our previous characterization of these mAbs demonstrated that the immunoreactivity of the antiregulatory domain mAbs, which includes M9, was sensitive to the PKC conformation, as shown by dot blot, Western blot, and immunoadsorption assays (38). We have also shown that our antiregulatory, but not the anticatalytic, domain mAbs inhibit [^{3}H]phorbol dibutyrate (PDBu) binding. This inhibition was sensitive to the phospholipid concentration used in the binding assay, suggesting that the epitope of M9 may be at or near the phospholipid binding site. The immunofluorescent staining with M9 suggests that PMA binding to PKC may lead to a conformational change of the regulatory domain such that the epitope recognized by M9 is no longer accessible, producing a different staining pattern than that of the anticatalytic domain mAbs. Masking of mAb sites is not unusual; for example, similar results have been observed in immunofluorescent staining of the fibronectin receptor in chick embryo fibroblasts (2). In cells in which extracellular matrix components are bound to the fibronectin receptor, polyclonal antibodies recognizing multiple epitopes produce different staining patterns than mAbs with epitopes which map to the ligand-binding site of the fibronectin receptor. Presumably, binding of the matrix components to the receptor reduces the accessibility to the mAbs, but not the polyclonal antibodies, resulting in the different staining patterns. Characterization of the epitopes recognized by M4, M7, and M9, as well as a better understanding of the relative affinities of each of the mAbs for PKC, is required for a full explanation of the observed differences in staining patterns.

Our results demonstrate that PMA treatment decreased the level of PKC in the cytosol with a concomitant increase in membrane PKC. This treatment also increased the level of PKC in the cell nucleus. In the immunofluorescence experiments with control cells, the nuclei were clearly visible due to an apparent lack of staining. These immunocytochemical results agreed closely with our biochemical analyses, since immunoblotting and PKC activity assays also demonstrated little or no nuclear PKC. The immunofluorescent staining of PMA-treated cells suggested that PKC might be localized to the nucleus after activation, since the staining covered the cell surface, including the nuclei. Subsequently, we showed by three criteria that PMA treatment specifically increased PKC in the nucleus: (*a*) immunofluorescent staining in isolated nuclei increased; (*b*) Western blots showed that our mAb detected only one protein, the 82-kD PKC, whose level increased in nuclear lysates from PMA-treated cells; and (*c*) PKC activity increased in nuclear lysates. Our results are the first demonstration using three different criteria that the activity and amount of a specific isoform, Type 3 PKC, increases in the nuclei of 3T3 cells. The brevity of the treatment period suggests that this is a rapid, primary event in PKC activation. Although our Western blot experiments demonstrated that PKC localized to the nuclear envelope, we do not yet know whether the PKC is located on the inside or the outside of the nucleus. Electron microscopy experiments will help resolve this issue. Our further experiments will also address whether agents, such as platelet-derived growth factor, which produce diacylglycerols in NIH 3T3 cells, will also result in PKC nuclear localization.

Evidence for or against nuclear PKC localization has been sought in several different cell types and tissues. The results of these studies have been diverse, perhaps reflecting the wide range of cell types and experimental protocols used, which include both reconstituted systems as well as intact cells (5, 34, 44). In some cases, no evidence for nuclear PKC could be found. Halsey et al. (17) showed increased perinuclear staining in PMA-treated 3T3-L1 cells using a polyclonal antibody to PKC. However, PKC activity was associated only with nonnuclear membranes and not with the nuclear fraction. Jensen and Sando (27) were unable to demonstrate either [^{3}H]PDBu binding or PKC activity in nuclei of either untreated or phorbol ester–treated EL4 mouse thymoma cells. In contrast, evidence for nuclear PKC has been demonstrated in other cell types. Nuclei isolated from bryostatin-treated HL-60 cells bind [^{3}H]PDBu (36) and Fields et al. (13) reported that short-term treatment with bryostatin, but not PDBu, increases calcium-dependent kinase activity in the nucleus of HL-60 cells. Kiss et al. (34) reported that PMA treatment of HL-60 cells for 2 d increases nuclear PKC. In B lymphocytes, both Ia-binding ligands and cAMP increase nuclear PKC activity and PKC levels (4). Thomas et al. (62) used a polyclonal anti–PKC Ab to show increased nuclear PKC levels in PMA-treated NIH 3T3 cells. The antibodies used in these last two studies were not characterized as to their type specificity. We found in our studies that immunocytochemical staining of the nucleus in PMA-treated cells was dependent on the mAb we used. Thus, immunocytochemical demonstration of PKC in localization studies may be highly dependent on the antibodies used and the epitopes that they recognize. Overall, these results are consistent with the hypothesis that the localization of PKC to the nucleus may be cell and/or isotype specific.

Our fractionation studies demonstrated clearly that Type 3 PKC is located in the nuclear envelope. This fraction has been shown to consist of the inner and outer nuclear membranes, pore complexes, and the lamina, a network of lamin

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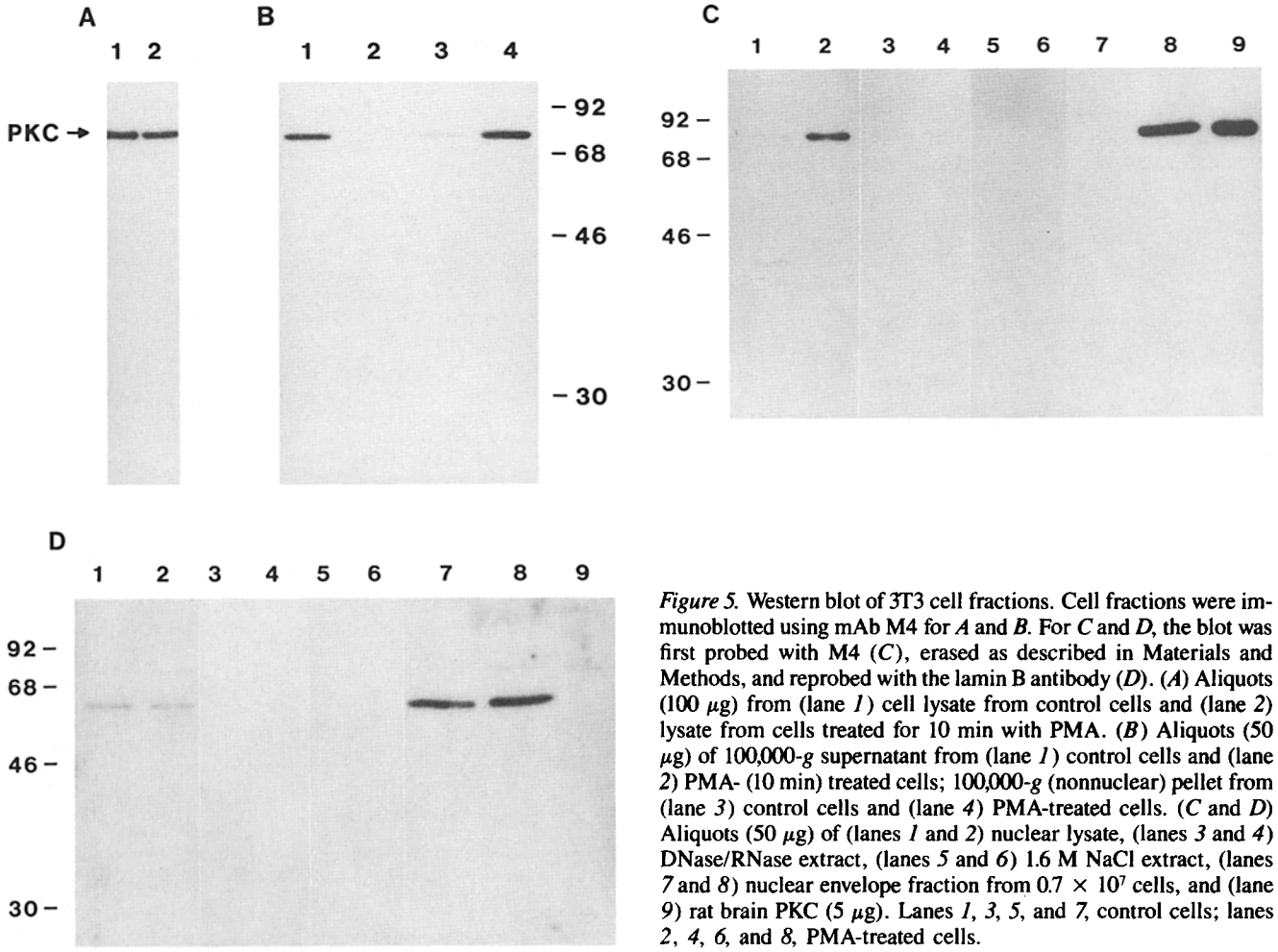

Figure 5. Western blot of 3T3 cell fractions. Cell fractions were immunoblotted using mAb M4 for *A* and *B*. For *C* and *D*, the blot was first probed with M4 (*C*), erased as described in Materials and Methods, and reprobed with the lamin B antibody (*D*). (*A*) Aliquots (100 μg) from (lane *1*) cell lysate from control cells and (lane *2*) lysate from cells treated for 10 min with PMA. (*B*) Aliquots (50 μg) of 100,000-*g* supernatant from (lane *1*) control cells and (lane *2*) PMA- (10 min) treated cells; 100,000-*g* (nonnuclear) pellet from (lane *3*) control cells and (lane *4*) PMA-treated cells. (*C* and *D*) Aliquots (50 μg) of (lanes *1* and *2*) nuclear lysate, (lanes *3* and *4*) DNase/RNase extract, (lanes *5* and *6*) 1.6 M NaCl extract, (lanes *7* and *8*) nuclear envelope fraction from 0.7 × 10^7 cells, and (lane *9*) rat brain PKC (5 μg). Lanes *1*, *3*, *5*, and *7*, control cells; lanes *2*, *4*, *6*, and *8*, PMA-treated cells.

proteins between the inner nuclear membrane and chromatin (32, 45). Lamin B has been shown to bind intermediate filaments such as vimentin, and several researchers have suggested that the cell surface is linked to the nucleus via intermediate filament attachments, forming a continuous cellular network (14, 16). The nuclear envelope also contains a number of enzymes, including RNA-activated NTPase (58), cyclooxygenase, isoforms of cytochrome P-450, and phosphoprotein phosphatases (42, 54). The localization of several different receptors, such as steroid, thyroid and insulin has been reported, as well as the demonstration of polyphosphoinositide metabolism, suggesting that the nuclear envelope may be a dynamic structure involved in signal transduction pathways (7, 39, 66).

The exact role of PKC in the nucleus is not yet known. Phorbol esters affect the transcription of many genes, including *c-fos*, plasminogen activator, ornithine decarboxylase, and interferon (9, 28, 29, 37). PMA-stimulated phosphorylation of a variety of nuclear proteins has been reported, including histones (3, 51), lamin B (13, 19), matrix proteins (41), and DNA topoisomerase II (55). Most of these studies have used reconstitution systems, and only some of these proteins, such as lamin B, are located in the nuclear envelope. Thus, the physiological PKC nuclear substrates have not been identified. The PMA-induced phosphorylation of intranuclear proteins such as histones may result from the action of a secondary kinase, or perhaps PKC can associate with other nuclear compartments. Using a short duration of PMA treatment, we found localization only in the nuclear envelope fraction. Whether longer PMA treatment or other PKC activators result in similar localization is an area of further investigation. The exact mechanism by which PKC-induced changes in phosphorylation leads to alterations in gene expression is unknown. PKC-stimulated in vitro phosphorylation of RNA polymerase II results in an increase in binding to GTP and an increase in the initial rate of RNA synthesis (6). This may be one way by which PKC could alter gene expression. Phosphorylation and activation of enhancer proteins that bind to phorbol ester responsive elements in DNA could serve as another regulatory mechanism (8, 10, 11, 48, 52, 53, 61). Our results suggest that Type 3 PKC may be one of the isozymes involved in these processes.

Our results in REF52 cells demonstrate PKC localization to the specialized area of the cell surface known as the adhesion plaque (26). Like the nuclear envelope, the plaque is also a dynamic structure associated with signal transduction. Taken together, these data in NIH 3T3 and REF52 cells showing Type 3 PKC association with cytoskeletal and nuclear components provide a framework for understanding function. In future experiments, we will determine if PKC

substrates are found in these areas, and furthermore, if phosphorylation can be blocked with Type 3 PKC-specific inhibitors such as M4.

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