Immunocytochemical Evidence for Translocation of Protein Kinase C in Human Megakaryoblastic Leukemic Cells: Synergistic Effects of Ca^{2+} and Activators of Protein Kinase C on the Plasma Membrane Association

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Abstract. Immunological analysis using monoclonal antibodies against subspecies of protein kinase C revealed the predominant expression of the isozyme, type II, in human megakaryoblastic leukemic cells. We investigated the effects of phorbol diester 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), the Ca^{2+} ionophore ionomycin and synthetic diacylglycerol 1-oleoyl-2-acetylglycerol (OAG) on the immunocytochemical localization of protein kinase C in these cells. Indirect immunofluorescence techniques revealed the enzyme to be located in a diffuse cytosolic pattern, in the intact cells. When the cells were exposed to 100 nM TPA, the immunofluorescent staining was translocated from the cytoplasm to the plasma membrane. The translocation was protracted and staining on the membrane decreased in parallel with the Ca^{2+}, phospholipid-dependent protein kinase activity. Treatment of the cells with 500 nM ionomycin caused an apparent translocation comparable with that seen with TPA, however, this translocation was transient and most of the cytosolic staining was within 60 min. We also found that 30 μg/ml OAG did not have significant effects on distribution of the staining, but rather acted synergistically on the translocation with the suboptimal concentration of 100 nM ionomycin. A similar syngergism was also observed with 10 nM TPA and 100 nM ionomycin. These results obtained in situ provide evidence that intracellular Ca^{2+} and diacylglycerol regulate membrane binding of the enzyme in vivo.

It is generally accepted that Ca^{2+}-activated, phospholipid-dependent protein kinase (protein kinase C) plays an important role in transmembrane signaling (27). The enzyme is active in the presence of both Ca^{2+} and phospholipids. Diacylglycerol, which is transiently generated as a consequence of the receptor-mediated hydrolysis of inositol phospholipids, and tumor-promoting phorbol diester, which have a chemical structure similar to that of diacylglycerol, increase the affinity of the enzyme for Ca^{2+} and phospholipid (20). Several agonists including phorbol diesters (23) and those noted to promote inositol phospholipids hydrolysis, such as gonadotropin-releasing hormone (17), thyrotropin-releasing hormone (11), interleukin 2 (8), interleukin 3 (9), and certain antibodies (4) cause a translocation of protein kinase C activity from the cytosol to the particulate (membrane) fraction in various cells and cell lines, which seems to be involved in the activation of the enzyme.

Inositol phosphates, the other products of hydrolysis of inositol phospholipids, increase intracellular free Ca^{2+} ($[Ca^{2+}]_i$)[1] by releasing Ca^{2+} from intracellular Ca^{2+} stores (38). Ca^{2+} ionophore such as ionomycin affect the permeability of the cell membrane to Ca^{2+} and are pertinent for use in studies on the role of $[Ca^{2+}]_i$ in the cell regulation. Moreover, the synthetic diacylglycerol or phorbol diester has been noted in various cellular responses (27, 35), including platelet secretion (13, 20), lymphocyte activation (39), smooth muscle contraction (34), and cell proliferation and differentiation (40). Although in vitro studies suggested that protein kinase C is activated in a component of a quarternary complex consisting of the kinase, Ca^{2+}, diacylglycerol, and phospholipid associated with cellular membrane structure (12), direct demonstration of the distribution or redistribution of the enzyme has been technically difficult, and the Ca^{2+} ionophore or diacylglycerol-induced translocation of

1. *Abbreviations used in this paper*: $[Ca^{2+}]_i$, cytosolic free calcium concentration; OAG, 1-oleoyl-2-acetylglycerol.

protein kinase C was not clearly demonstrated, using subcellular fractionation.

Analysis of the cDNA clones of the enzyme indicated that protein kinase C is a complex of a gene family and multiple subspecies of the enzyme are expressed in mammalian tissue (6, 21, 22, 33). Brain protein kinase C is further resolved into three fractions, type I, type II, and type III, upon chromatography on a hydroxylapatite column (18, 19). More recently, we developed monoclonal antibodies, MC-1a, MC-2a, and MC-3a, which specifically react with these species of rabbit brain protein kinase Cs, respectively, and a distinct expression of the subspecies of the enzyme in different tissue and cells was revealed (16).

Using these monoclonal antibodies, we have identified the subspecies of protein kinase C expressed in human megakaryoblastic leukemic (MEG-01) cells (28–32), and compared the effects of a phorbol diester, Ca^{2+} ionophores, and a synthetic diacylglycerol on intracellular distribution of the enzyme, in situ. Our results demonstrate that Ca^{2+} ionophores induce a translocation of the enzyme from the cytoplasm to the plasma membrane and that the other two agents act synergistically on the $[Ca^{2+}]_i$-dependent translocation of the enzyme. Furthermore, these results suggest a biochemical mechanism for the synergism between Ca^{2+} ionophores and a synthetic diacylglycerol or a phorbol diester observed in various cellular responses.

Materials and Methods

Production of Monoclonal Antibodies against Protein Kinase C

The preparation and properties of monoclonal antibodies used in the present study were described in detail elsewhere (16). Briefly, protein kinase C was purified from rabbit brain as previously reported (14). The enzyme protein (50 μg) was injected subcutaneously to 4-wk-old BALB/c male mice with 100 μg lipopolysaccharide (LPS) in complete Freund's adjuvant. After 4 wk, the mice were boosted by subcutaneous injection of 100 μg antigen in 100 μg LPS. Titers of antibodies in the serum were determined by ELISA. 3 d after the final booster injection with 200 μg of antigen in 100 μg LPS, the splenocytes were isolated and fused with SP2/0 myeloma cells. The hybridomas were screened for anti-protein kinase C immunoglobulin secretion by ELISA, and the cloned cells ($5–10 \times 10^5$) were injected intraperitoneally to BALB/c mice pretreated with 6, 10, 14-tetramethylpentadecane, and the resulting peritoneal ascites was collected. The three clones, designated MC-1a, MC-2a, and MC-3a were thus developed.

Cells and Cell Culture

The establishment of a human megakaryoblastic leukemia cell line, designated MEG-01, and some properties of the cells such as the synthesis or expression of protein S, β-thromboglobulin, and thrombomodulin have been described (28–32). The cells were grown in RPMI 1640 medium supplemented with 10% FBS (Gibco Laboratories, Grand Island, NY), penicillin G (100 U/ml), and streptomycin (50 μM) at 37°C in a humidified atmosphere of 5% CO_2, 95% air. For the immunocytochemical study, the cells were cultured on coverslips at 3×10^5/ml in 10 ml of medium for 24 h. As described (28), approximately half the number of the cells adhered to the coverslips, with extending pseudopods. Since these cells were largest (>40 μm) among human leukemic cells and rich in cytoplasm, identification of the located immunofluorescence was readily facilitated.

Immunocytochemical Procedures

Each coverslip was rinsed in 10 mM PBS and moved into the reaction medium (145 mM NaCl, 1 mM $MgSO_4$, 5 mM KCl, 1 g/liter glucose, 20 mM Hepes, pH 7.4, 1 mM $CaCl_2$, or 1 mM EGTA) containing several agents dissolved in 0.1% DMSO. After treatment at 37°C, the coverslips were immediately immersed in 4% paraformaldehyde/0.1 M phosphate buffer (PB) for 30 min, rinsed in PB, and subsequently incubated in 0.1% Triton-X100/PB for 15 min, then rinsed three times with PBS. After the incubation with 5% normal goat serum for 20 min to block to nonspecific-binding sites, the coverslips were incubated with antibody solution (1:100 dilution of mouse ascitic fluid) for 60 min. Then they were rinsed three times with PBS, incubated with the second antibody, fluorescein-conjugated goat $F(ab')_2$ anti-mouse IgG (TAGO, Inc., Burlingame, CA) (1:50 dilution) for 60 min, rinsed, and the coverslips were mounted with glycerol containing 5% *n*-propyl galate. All these procedures were carried out at room temperature.

Subcellular Fractionation

Cells (2×10^7), incubated with 0.1% DMSO (control) or 100 nM 12-*O*-tetradecanoyl phorbol-13-acetate (TPA), were collected and washed with ice cold PBS, disrupted in 1 ml of buffer A (20 mM Tris-HCl, pH 7.4, 0.25 M sucrose, 2 mM EGTA, 50 mM 2-mercaptoethanol, 0.2 mM phenylmethylsulfonyl fluoride, 100 μg/ml leupeptin) by sonication for 20 s. Whole homogenates were centrifuged at 900 *g* for 5 min to remove the cells debris and nuclei and the supernatant was centrifuged at 105,000 *g* for 60 min. The resultant supernatant served as the cytosol fraction. The pellet was dissolved in the same volume of buffer A containing 1% Triton X-100 was then used as the particulate fracton. For the kinase activity assay, these fractions were applied to 1 × 0.5 cm DEAE-cellulose columns previously equilibrated with buffer B (20 mM Tris-HCl, pH 7.4, 2 mM EGTA, 50 mM 2-mercaptoethanol, 10 μg/ml leupeptin). The columns were washed with 15 vol of buffer B, and the enzyme eluted in batches with 1 ml of 0.15 M NaCl in buffer B. Aliquots (20 μl) were assayed for protein kinase C activity in a 0.2 ml reaction mixture containing 20 mM Tris-HCl, pH 7.0, 10 mM Mg acetate, 0.5 mM $CaCl_2$, 10 μM [γ-^{32}P]ATP, 40 μg histone III-S, 10 μg phosphatidylserine, as described (15). The specific activity was quantitated by subtracting the amount of radiolabel incorporated into histone, by using parallel assays in the absence of phosphatidylserine. It was confirmed that the activity was also sensitive to TPA (not shown).

Immunoblotting Procedure

Whole homogenates, cytosol, and particulate fractions of the cells (2.5×10^5 cells/lane) were subjected to SDS-PAGE in 10% gels, followed by transblotting the protein onto a nitrocellulose membrane (Bio-Rad Laboratories, Richmond, CA). Rabbit brain protein kinase Cs (type I, type II, and type III) were purified as described (18, 19). Briefly, the homogeneous preparation from rabbit brain cytosol fraction by DE-52 column chromatography, followed by butyl-Toyopearl chromatography, was further resolved into three forms, type I, type II, and type III by hydroxylapatite column chromatography (16). Each type of protein kinase C (1 μg/lane) was subjected to the gel as described above. After transfer, the membrane was rinsed with TPBS (PBS containing 0.05% Tween-20) and incubated with 2% normal horse serum for 20 min, then subsequently incubated with monoclonal antibodies (1:100 dilution) for 60 min. After rinsing with TPBS, the membrane was incubated with biotinylated horse anti-mouse IgG for 30 min, rinsed again, and further incubated with the avidin-horseradish peroxidase complex (Vector Laboratories, Inc., Burlingame, CA) for 30 min. The immunoreactive proteins were visualized with 0.06% (wt/vol) 4-chloro-1-naphthol and 0.02% H_2O_2.

Other Chemicals

TPA (Sigma Chemical Co., St. Louis, MO), 1-oleoyl-2-acetylglycerol OAG (Sedary Research Laboratories, Ontario, Canada), ionomycin, A23187 (Calbiochem-Behring Corp., La Jolla, CA) were dissolved in DMSO and stored at −20°C. [γ-^{32}P]ATP was obtained from ICN Biomedicals Inc., Irvine, CA.

Results

Immunoreactivity and Immunocytochemical Localization of Protein Kinase C in Intact MEG-01 Cells

We developed monoclonal antibodies against rabbit brain protein kinase C and obtained three lots, MC-1a, MC-2a, and MC-3a that reacted specifically with type I, type II, and type III subspecies of rabbit brain protein kinase C, respectively (16). Immunoblotting of whole homogenates of MEG-01

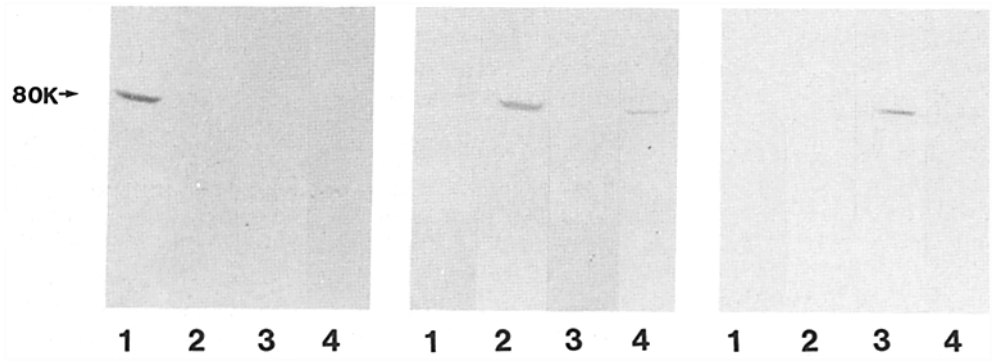

Figure 1. Immunoblotting with MC-1a, MC-2a, and MC-3a of whole homogenates of MEG-01 cells and three subspecies of rabbit brain protein kinase C. Whole homogenate (2.5×10^5 cells/lane) (lanes *4*) and type I (lanes *1*), type II (lanes *2*), type III (lanes *3*) rabbit brain protein kinase Cs purified with hydroxylapatite column chromatography (1 μg/lane) were subjected to SDS-PAGE in 10% gel, transferred to a nitrocellulose membrane, and reacted with each antibody, (*left*) MC-1a, (*center*) MC-2a, and (*right*) MC-3a. See Materials and Methods.

cells and the three subspecies of rabbit brain protein kinase C purified with hydroxylapatite column chromatography revealed that MC-2a, which reacted with type II–protein kinase C (80 kD) in rabbit brain and not with the other two subspecies of rabbit brain enzymes, recognized the 80-kD protein in MEG-01 cells (Fig. 1 *center*). Moreover, the reaction was specific to the protein and no other immunoreactive proteins were observed in whole homogenates of the cells. The 80-kD protein in the cells was only faintly detectable with MC-3a, which selectively recognized rabbit brain–type III enzyme (Fig. 1 *right*). No cross-reacting protein was observed in the cells with MC-1a, though it strongly reacted with type I–protein kinase C in rabbit brain (Fig. 1 *left*).

Similar results on the immunoreactivity of the enzyme in the cells were obtained with the indirect immunofluorescence method. MC-2a revealed a diffuse cytosolic distribution of the immunoreactive protein kinase C in intact MEG-01 cells (Fig. 2 *b*). Only a faint immunofluorescence staining was observed in cells incubated with MC-3a (Fig. 2 *c*), and no apparent staining was observed with MC-1a (Fig. 2 *a*). These results suggest that protein kinase c predominantly expressed in MEG-01 cells is immunologically identical to the type II subspecies of protein kinase C in rabbit brain.

Effects of TPA on the Localization of Protein Kinase C in MEG-01 Cells

As reported previously for various cells and cell lines (1), TPA induced a translocation of protein kinase C activity in the MEG-01 cells (Fig. 3). When the cells were treated with 100 nM TPA, the Ca^{2+}, phospholipid-dependent protein kinase activity in the cytosol fraction rapidly decreased. On the other hand, the activity in the particulate fraction increased and reached a maximum at 10 min, then gradually decreased. After 360 min, the total activity was reduced to 30% of that in the intact cells.

To examine the effect of TPA on the distribution of protein

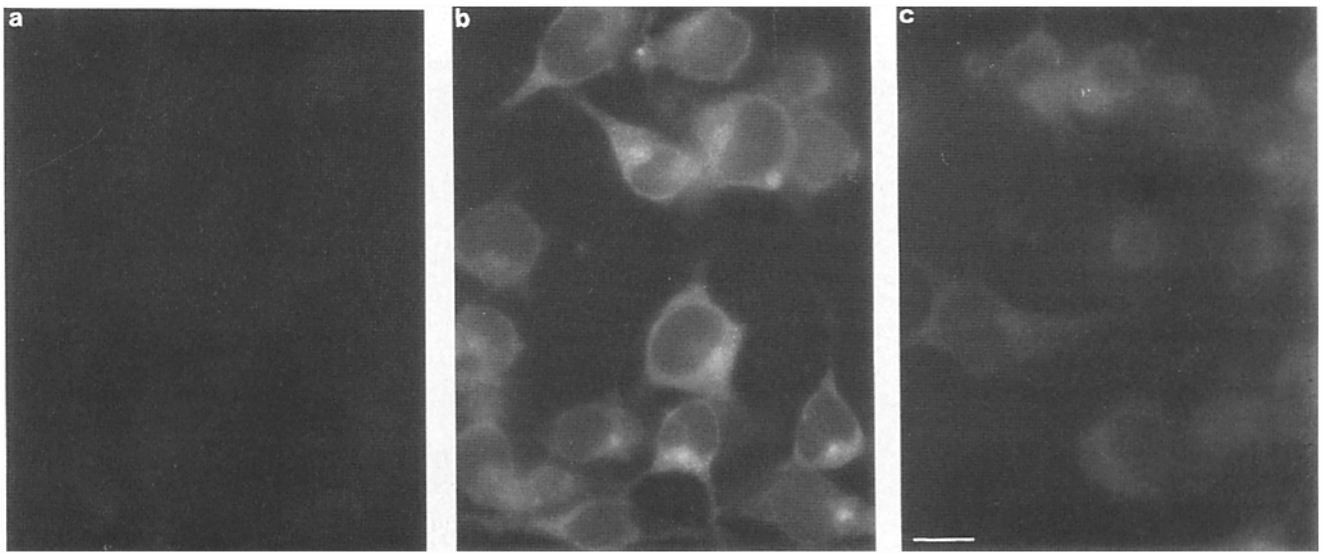

Figure 2. Immunoreactivity with MC-1a, MC-2a, and MC-3a in intact MEG-01 cells. The cells were incubated with each monoclonal antibody against protein kinase C–type I (MC-1a) (*a*), -type II (MC-2a) (*b*), -type III (MC-3a) (*c*), then stained by immunofluorescence, as described in Materials and Methods. Bar, 40 μm.

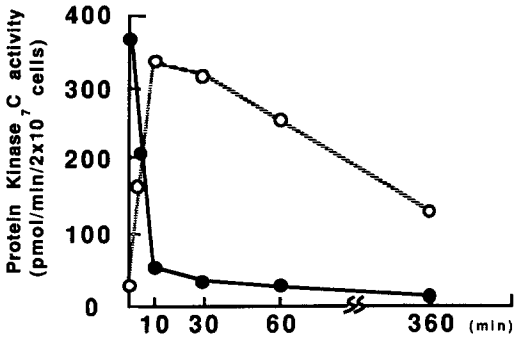

Figure 3. Protein kinase C activity in cytosol and particulate fractions of MEG-01 cells after exposure to TPA. MEG-01 cells (2×10^7) were incubated with 100 nM TPA at 37°C. At the times indicated, they were rapidly washed and the fractions were prepared. Each fraction was applied to DEAE-cellulose column and Ca^{2+}, phospholipid-dependent protein kinase activity in the eluates was measured as described under Materials and Methods. (●) Cytosol fraction; (○) particulate fraction.

kinase C and its immunoreactivity in situ, immunocytochemical studies were carried out using MC-2a, under the same conditions. The indirect immunofluorescence procedure revealed that 100 nM TPA rapidly decreases the cytosolic staining (Fig. 4). In 3 min, the cytosolic staining disappeared and in contrast, the immunofluorescence increased on some part of the plasma membrane, in ~80% of the cells (Fig. 4 *b*). After 10 min, all cells lost the cytosolic staining and the immunofluorescence staining was thoroughly translocated to the plasma membrane (Fig. 4 *c*). The membrane staining then gradually decreased and only a slight staining remained after 360 min (Fig. 4 *d*). DMSO (0.1%), as a carrier solvent, did not affect distribution of the enzyme (Fig. 4 *a*).

A similar translocation was observed on immunoblots of the cytosol and the particulate fractions (Fig. 5). When the cells were treated with TPA for 30 min, the 80-kD protein reacting with MC-2a in the cytosol fraction was decreased and it appeared in the particulate fraction. No other cross-reacting proteins were detected, even after the treatment with

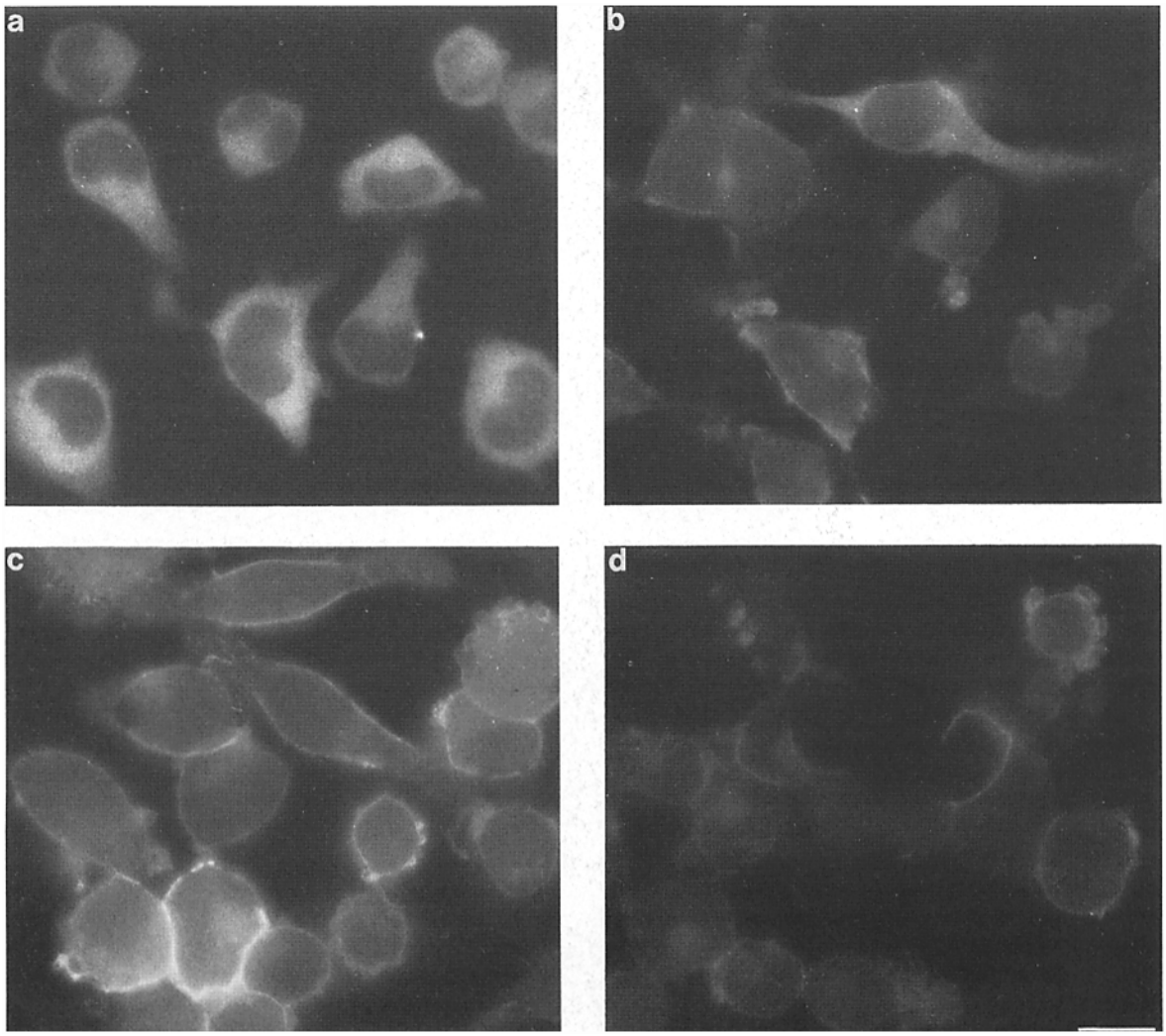

Figure 4. Effects of TPA on the immunofluorescent localization of protein kinase C in MEG-01 cells. The cells were treated with 0.1% DMSO for 10 min (control, *a*), or treated with 100 mM TPA at 37°C in the reaction medium for 3 (*b*), 10 (*c*), or 360 min (*d*), respectively. See Materials and Methods. Bar, 40 μm.

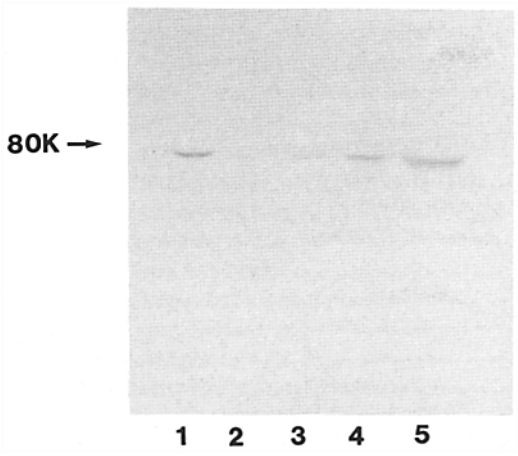

Figure 5. Immunoblot of cytosol and particulate fractions of MEG-01 cells treated with TPA. The cells were treated with 0.1% DMSO or 100 nM TPA for 30 min, then each fraction obtained under the same conditions as described in Fig. 3 was transferred to nitrocellulose membrane and immunoblotted with MC-2a. The cytosol fractions: (lanes *1* and *2*) and the particulate fractions (lanes *3* and *4*) of the cells treated with DMSO (lanes *1* and *3*) or with TPA (lanes *2* and *4*). (Lane *5*) Purified rabbit brain protein kinase C (type II).

TPA. These results show that the immunoreactivity of the protein kinase C, type II species in MEG-01 cells, parallels the Ca^{2+}, phospholipid-dependent protein kinase activity.

Effects of Ionomycin on Immunocytochemical Localization of Protein Kinase C in MEG-01 Cells

We further investigated the effects of Ca^{2+} ionophore ionomycin on the distribution of protein kinase C, immunocytochemically using MC-2a. As shown in Fig. 6, 500 nM ionomycin rapidly translocated the immunofluorescence staining from the cytoplasm to the plasma membrane, as did TPA. The maximum staining on the membrane was observed at ~3 min; that is earlier than that observed with 100 nM TPA (Fig. 6 *b*). Moreover, in contrast to TPA-induced translocation which was protracted and followed by the decrease in

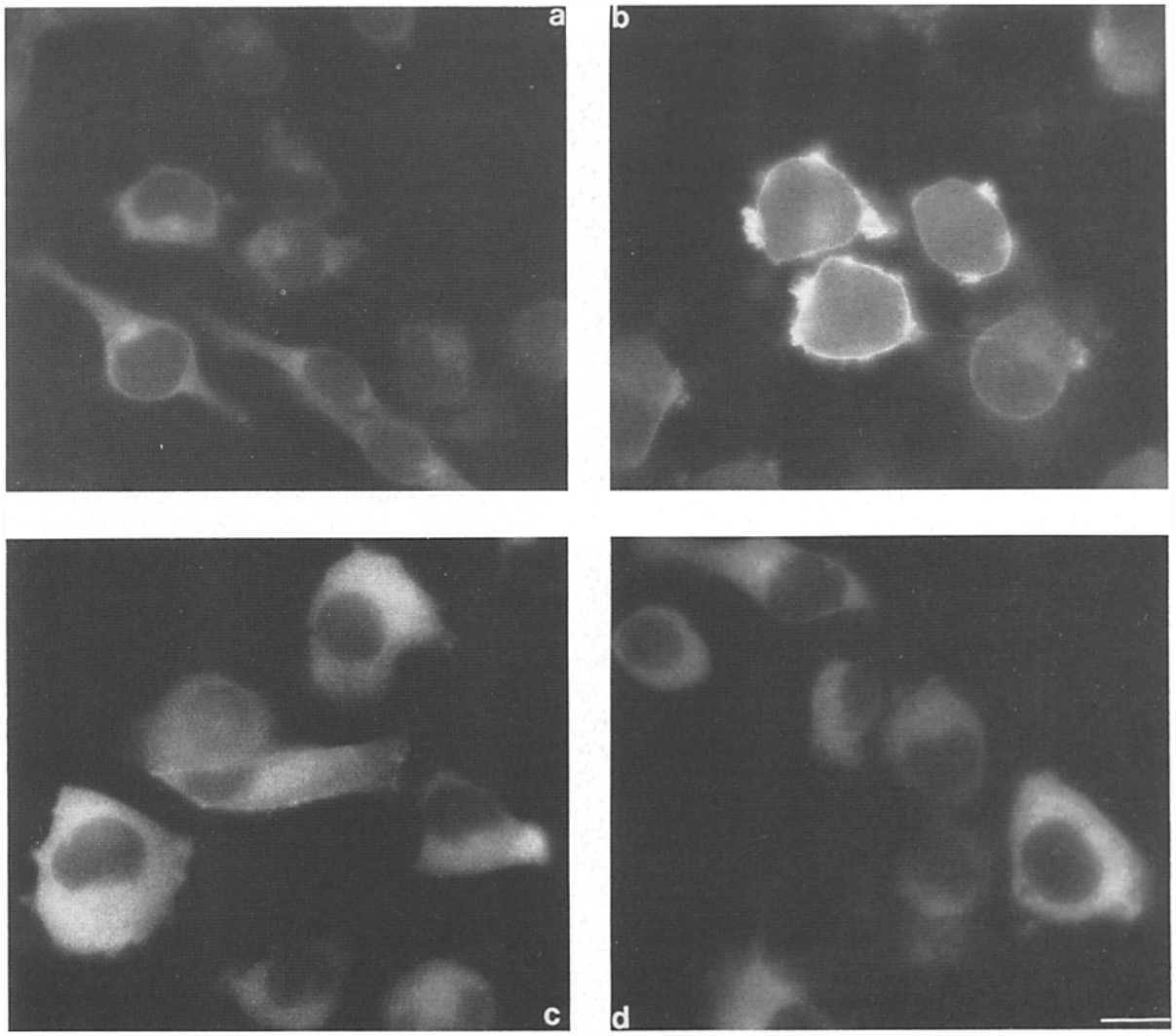

Figure 6. Effects of ionomycin on the immunofluorescent localization of protein kinase C in MEG-01 cells. The cells were treated with 500 nM ionomycin for 3 (*b*) or 60 min (*c*) in the presence of 1 mM $CaCl_2$. (*d*) Cells treated for 3 min in the presence of 1 mM EGTA. (*a*) Cells treated with 0.1% DMSO for 3 min. Bar, 40 μm.

staining of the plasma membrane, the ionomycin-induced translocation of the enzyme was transient and the cytoplasmic staining again increased and recovered to the pretreatment level within 60 min (Fig. 6 *c*). The effect of ionomycin was dose dependent and only a slight increase of staining on the membrane was observed at 100 nM (Fig. 7 *b*).

To confirm that the ionomycin-induced translocation is responsible for the increased $[Ca^{2+}]_i$, ionomycin was added to the reaction medium containing EGTA. In the presence of 1 mM EGTA, as shown in Fig. 6 *d*, no significant translocation was observed. These results indicate that extracellular Ca^{2+} is required for ionomycin to be effective, as detected by this method.

Synergistic Effect of OAG or TPA with Ionomycin on the Translocation of Protein Kinase C in MEG-01 Cells

Synthetic diacylglycerol, OAG, is suggested to intercalate into the cell membrane and to directly activate protein kinase C as a substitute for the physiologically generated diacylglycerol, as does TPA (20). When the MEG-01 cells were treated with 30 μg/ml OAG alone for 10 min, no significant translocation of the staining was observed (Fig. 7 *a*). As shown in Fig. 7 *b*, the low concentration of 100 nM ionomycin only slightly increased staining on the plasma membrane and most of the staining remained in the cytoplasm. However, when the cells were first treated with OAG for 10 min, the addition of 100 nM ionomyin induced a considerable increase of staining on the membrane and the cytosolic staining disappeared (Fig. 7 *c*). As observed in the translocation induced by a high concentration of ionomycin alone, the translocation was transient and the cytosolic staining recovered within 60 min (Fig. 7 *d*).

The synergistic effect on the translocation, in combination with ionomycin, was also observed with low concentration of TPA. No significant translocation of the immunofluorescence staining was observed with 10 nM TPA alone (Fig. 7 *e*). Preincubation with 10 nM TPA followed by the addition of 100 nM ionomycin caused an obvious translocation of the staining (Fig. 7 *f*). The translocation, however, was protracted and the translocated staining on the plasma membrane gradually disappeared in 360 min, as observed for 100 nM TPA-induced translocation (data not shown). These results suggest that OAG and TPA potentiate the Ca^{2+}-dependent binding of protein kinase C to the plasma membrane while the latter more strongly stabilizes the enzyme to the membrane and elicits a down-regulation of the enzyme.

Discussion

Protein kinase C is activated by phospholipid and diacylglycerol in the presence of Ca^{2+}, therefore, association of the enzyme with membrane is supposed to be required for activation of the enzyme. By using immunocytochemical technique, we investigated the effects of TPA, OAG, and the Ca^{2+} ionophore ionomycin on the intracellular distribution of protein kinase C, in situ.

Of the three monoclonal antibodies we developed to react against each respective isozyme of protein kinase C in the rabbit brain, MC-2a and MC-3a recognized subspecies of the enzyme in MEG-01 cells. The DNA sequences of each isozyme have a high homology among mammalian cells (6, 21), therefore, the preferential cross-reaction of the enzyme with MC-2a suggests that the type II protein kinase C is predominantly expressed in these cells.

Indirect immunofluorescence procedure using MC-2a revealed that TPA-induced translocation of immunofluorescence staining from the cytoplasm to the plasma membrane in MEG-01 cells (Fig. 4). Similar immunocytochemical evidence for the TPA-induced translocation of the enzyme was noted using a polyclonal antibody and different human leukemia HL-60 cells (37). In our study, we found that the translocated immunofluorescence was decreased in parallel with its Ca^{2+}, phospholipid-dependent protein kinase activity (Fig. 3) and the immunoreactivity of the 80-kD protein on immunoblotting (Fig. 5). Although it has been suggested that the enzyme translocated by TPA is modified into the Ca^{2+}, phospholipid-independent form and dissociated from the membrane by activation of Ca^{2+}-dependent neutral protease (26), we detected no significant change on the immunoblot of the cytosol fraction, except for a decrease in the immunoreactive 80-kD protein (Fig. 5). These results suggest that our antibody recognizes Ca^{2+}- and phospholipid-dependent form of protein kinase C.

We demonstrated here that ionomycin induced an apparent translocation of protein kinase C, comparable with evidence obtained using TPA. Similar results were obtained with a different Ca^{2+} ionophore A23187 (data not shown). The effective concentration exceeded 1 μM, possibly because of its lower specificity than ionomycin for Ca^{2+} (39). In contrast to the protracted translocation induced by TPA, the Ca^{2+} ionophore–induced translocation was transient. After recovery of the cytosolic staining, the same medium induced a similar translocation in the cells on other coverslips (not shown). The recovery of cytosolic enzyme did not seem to be due to the degeneration of Ca^{2+} ionophores or to a reproduction of the enzyme, but rather to a dissociation from the membrane, presumably by a decrease in $[Ca_2^+]_i$.

Ware et al. found that TPA also increase $[Ca^{2+}]_i$ of platelets, determined with the photoprotein aequorin (41). Our preliminary experiments using aequorin-loaded MEG-01 cells revealed that 100 nM ionomycin in the presence of 1 mM $CaCl_2$ induced a considerable increase in $[Ca^{2+}]_i$, however, the peak $[Ca^{2+}]_i$ induced by 100 nM TPA was much lower than that induced by 100 nM ionomycin with extracellular 1 mM EGTA (data not shown). These results and data from other studies (36) suggest that an increased $[Ca^{2+}]_i$ alone may not be responsible for the translocation of protein kinase C induced by TPA. Therefore, we investigated the effect of diacylglycerol or lower concentration of TPA combined with ionomycin.

OAG, although directly activating protein kinase C in vitro, as does TPA (20), had no significant effect on the distribution of immunofluorescence staining, even at a high concentration of 30 μg/ml, but did induce a prominent translocation of the enzyme when combined with suboptimal concentration of ionomycin. Moreover, a similar synergism on the translocation of protein kinase C was also observed with a low concentration of TPA and ionomycin. These results strongly suggest that diacylglycerol in the plasma membrane and cytosolic free Ca^{2+}, both of which are physiologically mobilized by the breakdown of inositol phospholipids, synergistically alter distribution of the protein kinase C from the cytoplasm to the plasma membrane.

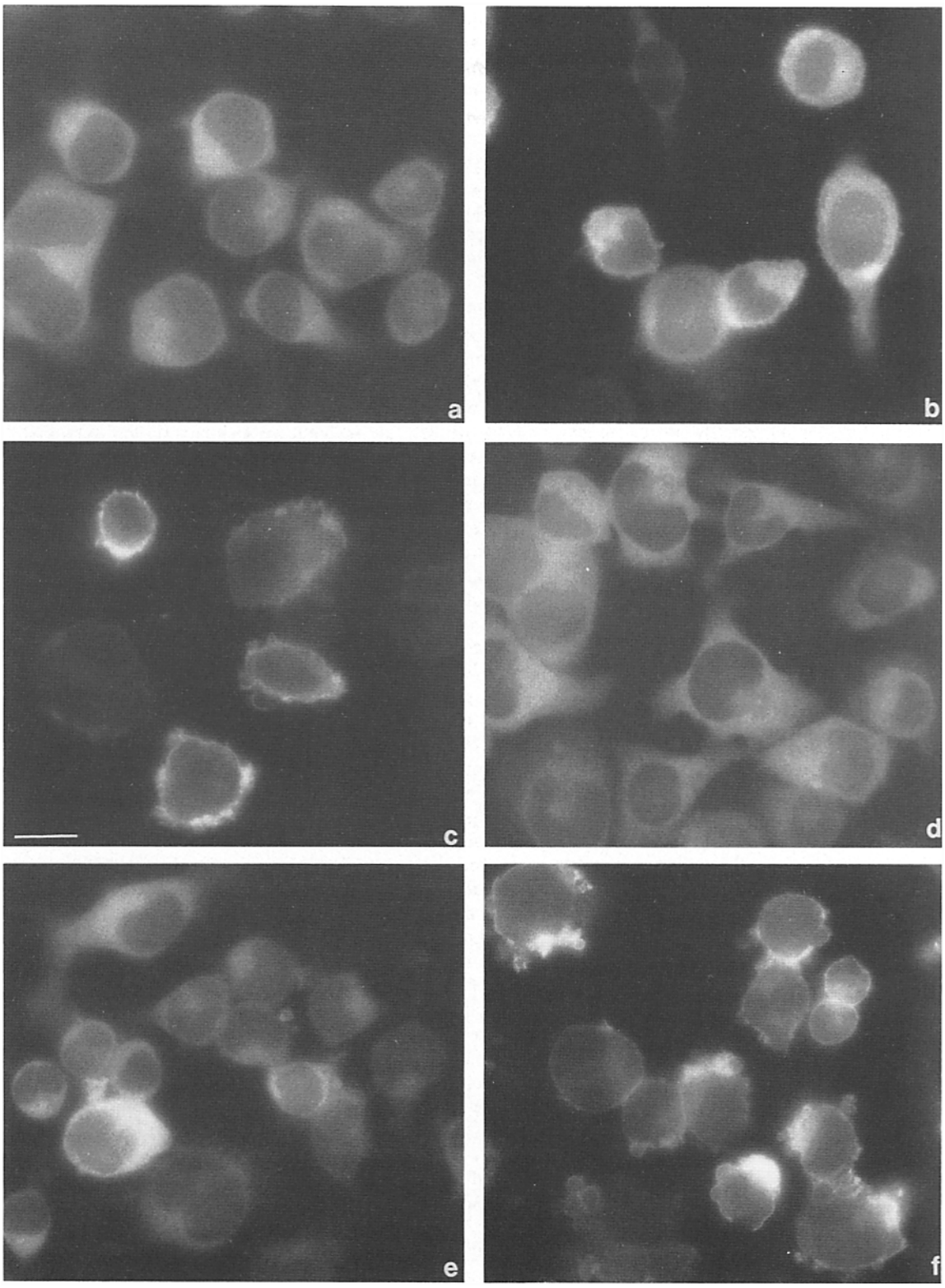

Figure 7. Synergistic effects of OAG or TPA with ionomycin on the translocation of protein kinase C. MEG-01 cells were treated with 30 μg/ml OAG (*a*) for 10 min, or 100 mM ionomycin for 3 min (*b*) alone, respectively. After preincubation with OAG for 10 min, 100 nM ionomycin was added to the medium and the preparations were further incubated for 3 (*c*) or 60 min (*d*). Likewise, other cells were preincubated with 10 nM TPA for 10 min (*e*), then additionally incubated for 3 min in the presence of 100 nM ionomycin (*f*). Bar, 40 μm.

The subcellular distribution or redistribution of the enzyme has been usually determined by measuring enzyme activity after cell homogenization or by assessing the radiolabeled phorbol diester binding. However, distribution of the enzyme between the cytosolic and particulate fraction apparently depends on the Ca^{2+} concentration in the homogenizing buffer (1). Moreover the presence of a Ca^{2+}-dependent protease may affect the enzyme activity (26, 27). In the latter method, the used phorbol diester, though it is used in a low concentration, may have a latent effect on the distribution of the enzyme. Although data on the time-dependent translocation of Ca^{2+}, phospholipid-dependent protein kinase activity after the exposure to TPA paralleled data obtained using immunocytochemical approaches (Fig. 3), we detected no significant translocation of the enzyme activity or immunoreactive protein on immunoblots from the cytosol to the particulate fraction of cells treated with ionomycin or OAG alone, or in combination, after homogenization (data not shown). A similar lack of success in exhibiting the Ca^{2+} ionophore or OAG-induced translocation of the Ca^{2+}, phospholipid-dependent enzyme activity has been reported (11, 25). Thus, the translocated enzyme may dissociate from the plasma membrane before the completion of subcellular fractionation. Another possibility is that the binding of protein kinase C to the plasma membrane, as induced by elevated $[Ca^{2+}]_i$ is weak, even in the presence of diacylglycerol, and the enzyme may be readily dissociated by homogenization of the cells by a Ca^{2+} chelator, such as EGTA. The immunocytochemical method is indeed useful for studying rapid and transient translocation of the enzyme, in response to extracellular stimuli.

Since OAG or TPA can directly activate protein kinase C in vitro at a low Ca^{2+} concentration comparable with $[Ca^{2+}]_i$ of unstimulated cells, synergistic effects of Ca^{2+} ionophore and OAG or TPA on cellular responses have been considered to be the simultaneous activation of two pathways: Ca^{2+}-dependent pathway (such as Ca^{2+}–calmodulin–dependent pathway) and protein kinase C–dependent pathway. On the other hand, Wolf et al. showed that Ca^{2+} induced purified protein kinase C to associate reversibly with erythrocyte ghost membrane and that phorbol diester enhanced the binding reaction (42, 43). Other in vitro studies also suggested that Ca^{2+} and diacylglycerol increase the affinity of the enzyme for phospholipids (3, 12). Despite these in vitro observations, there has been no in vivo evidence that Ca^{2+} ionophore or diacylglycerol induce such an apparent translocation of the enzyme as induced by TPA. Dougherty et al. reported an indirect evidence that A23187 increases phorbol diester binding affinity in intact phagocytes (7). In the present study, we obtained the direct evidence that "Ca^{2+} mobilization" alters the subcellular distribution of the enzyme itself and diacylglycerol increase the sensitivity of the binding reaction to the plasma membrane, thereby supporting the in vitro results. These observations also suggest another possible mechanism of synergism between Ca^{2+} ionophore and OAG (or TPA) noted in various cellular responses.

The loss of activity (5) and immunoreactivity (2) of the enzyme after a long term incubation with TPA has been termed down-regulation. Binding of the enzyme to plasma membrane has been thought to convert the enzyme to the proteolytically modified form that no longer binds to the membrane and is activated in the absence of Ca^{2+} and phospholipids (26). However our observations on the transient translocation induced by Ca^{2+} ionophores alone, or in combination with OAG indicate that $[Ca^{2+}]_i$ elevation and the resultant binding of the enzyme to the plasma membrane are not sufficient to down-regulate the enzyme, even in the presence of diacylglycerol. As the physiological increase of $[Ca^{2+}]_i$ and diacylglycerol in stimulated cells is apparently transient, we suggest that proteolytic activation of the enzyme may not be involved in physiological responses. Our recent study indicated that TPA induced terminal differentiation of MEG-01 and the subline, MEG-01s cells (30). Although ionomycin acted synergistically in this phenomenon, OAG was unable to mimic this effect, even in the presence of ionomycin (unpublished observations). Similar results have been noted in HL-60 cells (24, 40, 44). The protracted form of translocation of the enzyme caused by TPA alone may be significant for some biological events, such as cell differentiation.

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