

Temporal changes in intracellular distribution of protein kinase C during differentiation of human leukemia HL60 cells induced by phorbol ester

Zoltan Kiss, Eva Deli and J.F. Kuo

Department of Pharmacology, Emory University School of Medicine, Atlanta, GA 30322, USA

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Immunocytochemical methods were used to study protein kinase C (PKC) distribution in HL60 cells during the entire course of 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced differentiation. After an initial translocation of PKC from cytoplasm to plasma membrane, the enzyme was localized close to the nuclear membrane region at day 1 of TPA treatment. PKC was associated with nuclei at day 2 and with nuclei, cytoplasm and plasma membrane at days 3 and 5. Attachment of cells to substratum (day 2) was accompanied by increased phosphorylation of several nuclear proteins. At day 7, the differentiated cells became detached and PKC in these cells was largely cytoplasmic. In view of the crucial role of PKC in cell differentiation, it is expected that changes in its intracellular localization have physiological significance.

Differentiation; 12-*O*-tetradecanoylphorbol-13-acetate; Protein kinase C

1. INTRODUCTION

Tumor promoting TPA induces macrophage-type differentiation of human promyelocytic leukemia HL60 cells [1,2]. The primary target of TPA action is PKC [3] which occurs widely in animal cells [4]. Data obtained with the use of TPA-resistant cell lines [5–7] and various PKC inhibitors [8–11] suggest that PKC has a crucial role in the regulation of leukemic cell differentiation. The biochemical changes which trigger differentiation are still largely unknown. However, both topoisomerase II [12] and polymerase II [13] have been shown to serve as substrates for PKC, indicating that some nuclear functions are potentially regulated by this kinase. Increased

phosphatidylcholine (PC) metabolism in the plasma membrane has also been shown to be an important marker of differentiating HL60 cells [14]. All these suggest that phosphorylation of both plasma membrane and nuclear proteins by a TPA-stimulated mechanism is required for differentiation to occur. Both events can take place since rapid translocation of PKC to plasma membranes [15] as well as high affinity PKC binding sites and PKC immunoreactivity in isolated nuclei [16–18] were demonstrated.

In HL60 cells, TPA induces a rapid (within 10–30 min) translocation of the enzyme from cytoplasm to plasma membrane [19,20]. The present studies investigated possible long-term effects of TPA on PKC distribution. Proteases are known to transform a significant portion of membrane bound PKC to a lower molecular mass, irreversibly activated, form [21–23]. It is conceivable that this truncated PKC (PKM) will dissociate from membranes and redistribute to other intracellular sites more readily than the whole, more hydrophobic, molecule. However, present biochemical methods

Correspondence (present) address: Z. Kiss, National Institutes of Health, Bldg 36, Rm 1D 22, Bethesda, MD 20892, USA

Abbreviations: PKC, protein kinase C; PKM, proteolytically activated form of PKC; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PS, phosphatidylserine; PC, phosphatidylcholine

do not distinguish between PKM and other protein kinases which makes the study of possible intracellular redistribution of the former enzyme very difficult. To overcome this potential difficulty, in the present work we used immunostaining and immunofluorescence methods to demonstrate temporal changes in PKC distribution during the entire course of differentiation.

2. MATERIALS AND METHODS

2.1. Materials

TPA was purchased from Sigma; the Vectastain biotin-avidin-peroxidase kit was from Vector Laboratories; fluorescein-conjugated goat anti-rabbit IgG was from Cooper Biomedical; [γ - 32 P]ATP was prepared by the method of Post and Sen [24]. Polyclonal anti-PKC antisera [25] was kindly pro-

vided by Peggy R. Girard of this laboratory. PKC was purified from pig brain as described for the heart enzyme [26]. Human promyelocytic cell line HL60 [27] was cultured as described [28].

2.2. Immunocytochemical localization of PKC

Cells from log phase were diluted into plastic dishes (2×10^5 /ml) incubated with 1 nM TPA for up to 7 days. Suspended cells (untreated or treated with TPA for 1 or 7 days) were first pelleted using a cytospin (5 min) and fixed. Attached cells (treated with TPA for 2, 3 and 5 days) were directly fixed in the plastic dishes. Fixation (30 min) was performed with 4% (w/v) paraformaldehyde and 0.1% glutaraldehyde in 50 mM Tris-HCl (pH 7.5) followed by washing (7.5 min) with 0.2% (w/v) Triton X-100. The immunostaining procedure (fig.1) was the same as described [18–20]. For immunofluorescence visualization of PKC (fig.2), fixed cells were pretreated with polyclonal antisera and stained with fluorescein-conjugated goat anti-rabbit IgG [18–20]. Preimmune serum was used as control which exhibited little or no immunoreactivity [18,25].

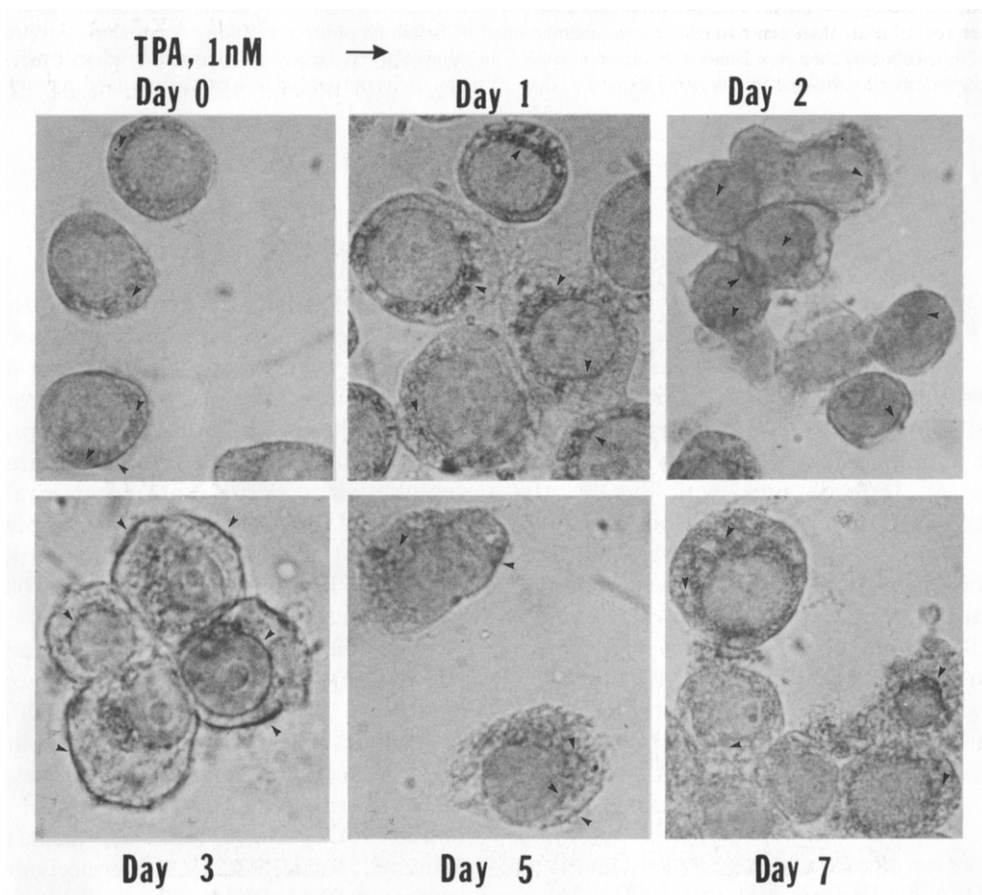


Fig.1. Redistribution of PKC during differentiation of HL60 cells induced by TPA as determined by immunoperoxidase method. The cells were incubated with 1 nM TPA for up to 7 days. The cells at days 0 and 1 were in suspension (undifferentiated), at days 2, 3 and 5 were attached to substratum (differentiating), and at day 7 became detached and were in suspension (differentiated). Visualization of PKC was performed by the immunoperoxidase technique (see section 2). Arrow heads indicate the sites of immunoreactivity.

2.3. Endogenous protein phosphorylation in nuclei from control and attached cells

Nuclei fractions from control and attached (day 2 after TPA treatment) cells were prepared as described [29]. Briefly, the cells were suspended in homogenization buffer (10 mM Tris-HCl, pH 7.5, 2.5 mM $MgCl_2$, 1 mM PMSF, 50 μ M leupeptin and 0.3 M sucrose) and passed through a syringe (27G 5/8 needle) 15–20 times to rupture cells. Homogenates were centrifuged at $1000 \times g$ for 10 min and the resulting pellets (nuclear fraction) were washed twice with the homogenization buffer. The pellets were dissolved in homogenization buffer containing 0.1% Triton X-100 (instead of sucrose), sonicated for 1 min on ice and kept for 20 min on ice before use. The incubation medium (0.15 ml) for the endogenous protein phosphorylation assay contained 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 25 μ M [γ - ^{32}P]ATP (4800 cpm/pmol), 0.1% Triton X-100, 0.5 mM PMSF, 25 μ M leupeptin, 50–60 μ g nuclear proteins and, if present, 10 μ g of phosphatidylserine (PS), 10 μ M $CaCl_2$, 100 nM TPA and 0.2 μ g purified PKC. Incubations were carried out in a shaking water bath for 5 min at 30°C. Reactions were terminated by adding 1.4 ml ice-cold acetone; samples were kept on ice for 15 min, then centrifuged and the pellets were solubilized by incubating them for 5 min in a solution containing 2% sodium dodecyl sulfate, 20% glycerol, 10 mM Tris-

HCl, pH 7.5 and 1 mM dithiothreitol. Equal amounts of proteins (40 μ g) were applied onto 10% polyacrylamide gels. Electrophoresis and detection of phosphoproteins by autoradiography were performed as described [7].

3. RESULTS

We have reported [19,20] that TPA (10–200 nM) caused a rapid (within 10–30 min) translocation of PKC from cytoplasm to plasma membrane in HL60 cells. In the present studies, we examined the patterns of PKC redistribution in HL60 cells during the entire course of differentiation induced by a low concentration (1 nM) of TPA. At day 0 of TPA treatment (i.e. no treatment), the PKC immunostaining in the cells was largely cytoplasmic, but it became more concentrated around nuclear membrane or perinuclear region at day 1 (fig.1). PKC was then associated with the nuclear membrane region and, to a lesser extent, in plasma membrane at day 2, and was

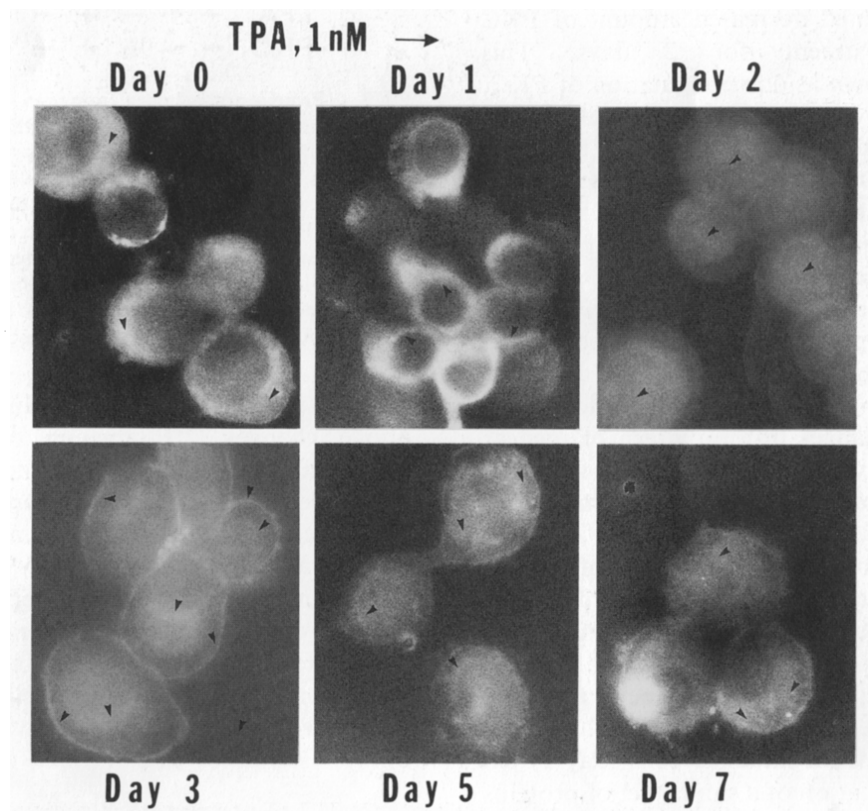


Fig.2. Redistribution of PKC during differentiation of HL60 cells induced by TPA as determined by immunofluorescence technique. The treatment and characteristics of the cells were essentially the same as indicated in fig.1. Visualization of PKC was performed using the immunofluorescence technique (see section 2). The arrow heads indicate the sites of immunoreactivity.

heavily localized in both plasma and nuclear membranes at day 3. By day 5, most of the immunoreactivity was detected in cytoplasm and nuclear membrane although some staining was also evident in plasma membrane. Most of the cells (about 70%) became attached to substratum (characteristic of differentiating cells) during days 2–5, and immunocytochemical micrographs of PKC in these attached cells were shown here. Most of the staining was found in the cytoplasm at day 7, when the cells became fully differentiated and detached from the substratum (fig.1). Patterns of temporal changes in PKC redistribution during the course of differentiation of HL60 cells, similar to those shown above in fig.1, were also observed in a separate series of experiments using immunofluorescence technique to visualize the enzyme (fig.2). We should note here that in many attached cells, treated with TPA for 24–48 h, less immunoreactive material seemed to be present than in control cells. Fig.2 shows a population of cells which contained decreased amount of PKC after TPA treatment for 2 days. This phenomenon, known as down-regulation of PKC, may be due to protease-catalyzed loss of the enzyme which often occurs in response to TPA-treatment [21–23]. In five experiments, performed similarly to that reported in fig.2, we have not observed significant down-regulation of the enzyme at later stages of differentiation.

Because nuclear localization of the PKC immunoreactivity appeared to be the most obvious at day 2 of TPA treatment (figs 1 and 2), we chose the attached cells at this stage of differentiation to examine possible changes in the pattern of nuclear protein phosphorylation. It was observed that phosphorylation of nuclear proteins from control cells was not stimulated by PS/Ca²⁺/TPA (fig.3, lanes 1 and 2), but phosphorylation of several other proteins (notably the 98, 85, 80, and 45 kDa species) was augmented by PS/Ca²⁺/TPA in the presence of exogenous PKC (fig.3, comparing lane 4 with lane 3), suggesting a deficiency in nuclear PKC in control cells to phosphorylate these proteins. The nuclei from attached cells (day 2) exhibited phosphorylation of a similar set of proteins shown to be phosphorylated by exogenous PKC in the nuclei from control cells (fig.3, comparing lane 5 with 4); however, several proteins, especially the 98, 71, and 48 kDa species, were more highly

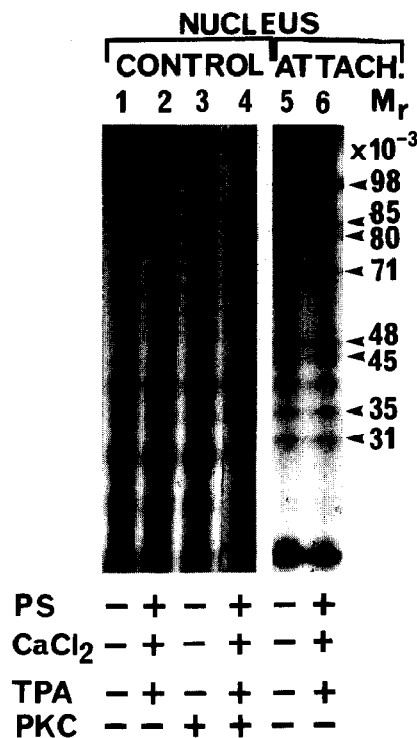


Fig.3. Protein phosphorylation in the nuclei from undifferentiated and differentiating HL60 cells. The nuclei from undifferentiated (suspended) cells (lanes 1–4) untreated with TPA and differentiating (attached) cells 2 days after treatment with 1 nM TPA (lanes 5 and 6) were incubated with [γ -³²P]ATP in the absence or presence of PS (10 μ g), CaCl₂ (10 μ M) and TPA (100 nM), as indicated. Purified pig brain PKC (0.2 μ g) was also present in certain incubation mixtures (lanes 3 and 4). Detailed procedures for purification of nuclei and protein phosphorylation were described in section 2.

labelled in the attached cells. In addition to the above changes, ³²P-labelling of several lower molecular mass proteins prominent in the control cell nuclei (fig.3, lanes 1–4), were undetectable in the attached cell nuclei (fig.3, lane 5). Using H1 histone as exogenous substrate, we found about 20-fold more PS/Ca²⁺/TPA-dependent PKC activity in attached cell nuclei, compared to control cell nuclei (not shown). In spite of this, protein phosphorylation in attached cell nuclei was not stimulated further by PKC activators (fig.3, lane 6).

4. DISCUSSION

To our knowledge this is the first report which describes sequential changes in PKC distribution

during the entire course of HL60 cell differentiation. Activation of PKC, the only hitherto recognized cellular target of phorbol esters, is crucial for the macrophage-type differentiation of leukemic cells, induced by TPA [5–11]. Although this suggests that the observed alterations in PKC localization are physiologically important, a causal relationship between PKC distribution and HL60 cell differentiation remains to be proven.

During the course of this and a previous study [20], we observed that 30 min treatment of cells with 100 nM TPA (followed by washing of cells) was sufficient to induce both PKC translocation from cytoplasm to plasma membrane (immediate effect) and, within 48 h, cell attachment. At low TPA concentration (1 nM), similar effects on both cell attachment and PKC translocation were observed if cells were treated for at least 4 h. It appears that the length of TPA treatment required for the induction of cell differentiation strictly correlates with the time needed for the translocation of PKC to plasma membrane, suggesting a relationship between the two events. We have only limited information about the possible biochemical mechanism(s) which may underlie this relationship. We and others observed significant changes in the degradation and synthesis of PC [14,20,28] and PS [29], following the early partitioning of PKC to plasma membrane in response to TPA. Modification of membrane lipid content, and thereby the membrane structure, is likely to be a precondition for cell attachment.

Translocation of PKC to the nucleus at day 2 of TPA treatment was associated with increased ^{32}P -labelling of several proteins. Increased phosphorylation of most of these proteins was also observed when purified PKC was added to control cell nuclei. However, the basal endogenous phosphorylation in attached cell nuclei was not modified by $\text{PS}/\text{Ca}^{2+}/\text{TPA}$, suggesting that either a truncated PS/Ca^{2+} -independent PKC, or another protein kinase (or both) was involved. Mapping of phosphorylated sites of these proteins, a tool to investigate protein kinase specificity, will be required to identify the enzymes involved. Phosphorylation of several lower molecular mass nuclear proteins, prominent in control cells, were undetectable in attached cells. While the reason for this phenomenon is not known, one should be aware that ^{32}P -labelling of endogenous proteins in

vitro may not reflect the in vivo situation. It is important, therefore, to study phosphorylation of nuclear proteins in the intact cells as well. In preliminary experiments we prepared the nuclei after incubating the cells with $^{32}\text{P}_i$ for 8 h, and found that in attached cells the 98, 85 and 80 kDa proteins were the most highly labelled, and that the lower molecular mass proteins remained poorly labelled.

While the translocation of PKC to cell nucleus is clearly accompanied by an altered protein phosphorylation profile, we cannot relate these changes to specific biochemical mechanisms involved in differentiation. It is known, however, that various markers of the macrophage-type differentiation, such as superoxide production, non-specific esterase activity and the cell surface marker Mo1 appear only 48–72 h after TPA treatment [30]. Conceivably, the nucleus-associated PKC may be involved in the regulation of those genes responsible for the production of these differentiation markers. In this respect, it seems worth mentioning that apparently more PKC was accumulated in nuclei of attached cells than that required for endogenous protein phosphorylation, suggesting that the enzyme may have other functions as well. Recent evidence indicates that DNA binding proteins contain a common structural motif, called 'zinc finger', which is involved in binding to DNA [31]. Interestingly, PKC also contains this putative DNA binding domain, and it has been suggested that PKC may directly bind to DNA and modulate gene expression [32].

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