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Sustained activation of protein kinase C is essential to HL-60 cell differentiation to macrophage

(dioctanoylglycerol/tumor promoter)

HIROAKI AIHARA*†, YOSHINORI ASAOKA‡, KIMIHISA YOSHIDA*, AND YASUTOMI NISHIZUKA*§

*Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan; and ‡Biosignal Research Center, Kobe University, Kobe 657, Japan

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ABSTRACT **Although a single dose of phorbol 12-myristate 13-acetate (PMA) allowed HL-60 cells to differentiate to macrophages, a single dose of membrane-permeant diacylglycerol (DAG), 1,2-dioctanoylglycerol (1,2-DiC_8), was normally insufficient to differentiate these cells. These cells metabolized 1,2-DiC_8 very rapidly, and 1,2-DiC_8 available to protein kinase C (PKC) activation was removed from the incubation medium at a rate proportional to cell density. However, increasing the duration of exposure of HL-60 cells to this DAG either by its repeated addition or by decreasing the cell density greatly enhanced their differentiation to macrophages as measured by CD11b expression. During this differentiation induced by DAG, neither measurable translocation nor depletion (down-regulation) of PKC was observed. When the cells were exposed to PMA, on the other hand, some PKC subspecies were instantaneously translocated to membranes and subsequently disappeared very quickly, whereas the α-subspecies was decreased to the level of ≈60% of the resting cell, but thereafter its activity was maintained at a nearly constant level in membranes. After ≈4 hr, the PKC subspecies, once depleted, reappeared gradually in the membrane fraction. The results suggest that sustained activation of PKC is essential to differentiation of HL-60 cells to macrophages, and depletion of the enzyme is not needed. Perhaps translocation of PKC represents an extreme state of the active form of the enzyme, which may result from PMA action, and the α-subspecies presumably plays a key role in HL-60 cell differentiation.**

The HL-60 cell line is frequently used as a model system for investigation of the mechanism of cell differentiation, since retinoic acid and several other chemicals lead this cell to differentiate to a granulocyte, whereas phorbol 12-myristate 13-acetate (PMA) promotes the cell to differentiate to a macrophage (for a review, see ref. 1). For the actions of PMA and membrane-permeant diacylglycerols (DAGs) such as 1,2-dioctanoylglycerol (1,2-DiC_8), several conflicting reports have appeared, showing that PMA induces HL-60 cell differentiation to macrophages, whereas the DAG does not (2–4). The present studies were undertaken to examine whether the biological actions of PMA and 1,2-DiC_8 on HL-60 cells are distinctly different, both quantitatively and qualitatively. The results described herein will indicate that the apparently different actions of PMA and 1,2-DiC_8 may result from the fact that 1,2-DiC_8 is metabolized very fast and thus protein kinase C (PKC) may be activated only transiently, whereas PMA is hardly metabolized and thus PKC remains active for a prolonged period of time. Sustained activation of this enzyme appears to be a prerequisite essential to cause cell differentiation. Translocation and depletion (down-regulation) of several subspecies of PKC in HL-60 cells are also investigated.

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MATERIALS AND METHODS

Materials. HL-60 cells donated by J. Minowada (Fujisaki Cell Center, Hayashibara Biochemical Laboratories, Okayama) were maintained at a cell density between 0.5 and 2×10^6 cells per ml as a suspension in RPMI 1640 medium supplemented with 5% fetal bovine serum (GIBCO) at 37°C in humidified 5% CO_2/95% air.

Chemicals. The membrane-permeant DAG, 1,2-DiC_8, was obtained from Nakarai Tesque, Kyoto. This preparation consists of ≈95% DL-1,2-DiC_8 and 5% 1,3-DiC_8 as estimated by TLC. The three isomers of DAG (1,2-*sn*-, 2,3-*sn*-, and 1,3-DiC_8) were gradually isomerized to an equilibrium state nonenzymatically in aqueous medium at room temperature, but 1,2-DiC_8 could be stored for at least 1 month in chloroform at −20°C without measurable isomerization or degradation. However, the 1,2-DiC_8 used for the present studies was a mixture of DL-isomers that could not be distinguished from each other by TLC. Radioactive 1,2-DiC_8 [DL-glycerol-1,2-di[1-^{14}C]octanoate (27 mCi/mmol; 1 Ci = 37 GBq)] was a product of Amersham.

PMA and ionomycin were products of Chensym Science Laboratories, Lenexa, KS, and Calbiochem–Behring, respectively. Other chemicals were obtained from commercial sources.

Determination of CD11b Expression. HL-60 cells were stimulated and incubated as specified in each experiment. After incubation, the cells were collected, washed, and resuspended in phosphate-buffered saline (PBS) at a density of 1×10^7 cells per ml, and treated for 30 min at 4°C with an appropriately diluted mouse monoclonal antibody against CD11b (Immunotech S.A., Marseille, France). After three washes, the cells were incubated with fluorescein-conjugated rabbit anti-mouse IgG (Seikagaku Kogyo, Tokyo) for 30 min at 4°C and then washed and resuspended in PBS. The fluorescence was determined by analysis of 3000 cells with a Showa Denko CS-20 flow cytometer.

Determination of 1,2-DiC_8. Metabolism of 1,2-DiC_8 was analyzed with DL-1,2-[^{14}C]DiC_8 as described (5). Briefly, radioactive 1,2-DiC_8 in chloroform was dried under a nitrogen stream and dispersed in PBS by vigorous mixing followed by sonication for 3 min at 0°C. HL-60 cells were suspended in RPMI 1640 medium at various cell densities as specified. The radioactive DiC_8 was added to the cell suspension at a final concentration of 100 μM ($2–3 \times 10^5$ cpm/ml). After incubation, lipids were extracted directly from the cell suspension by the method of Bligh and Dyer (6). 1,2-DiC_8 was separated by TLC with a solvent system composed of chloroform/acetone (96:4; vol/vol). The radioactive material corresponding to 1,2-DiC_8 was quantitated with a BAS-2000 Bioimage analyzer (Fuji photo film, Tokyo).

Abbreviations: PKC, protein kinase C; DAG, diacylglycerol; DiC_8, dioctanoylglycerol; PMA, phorbol 12-myristate 13-acetate.

†Permanent address: Department of Pediatrics, Kobe University School of Medicine, Kobe 650, Japan.
§To whom reprint requests should be addressed.

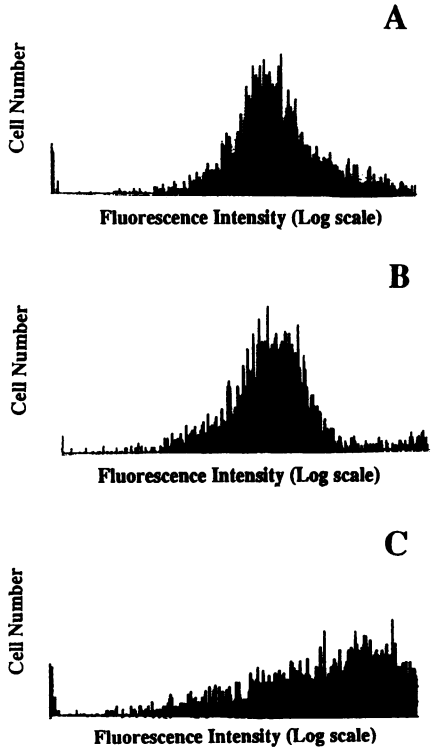

FIG. 1. CD11b expression in HL-60 cells treated with 1,2-DiC_8 and PMA. HL-60 cells (5×10^5 cells per ml) were treated with either 1,2-DiC_8 or PMA. After 48 hr, the expression of CD11b was quantitated as described. (*A*) No stimulation. (*B*) 1,2-DiC_8 (100 μM). (*C*) PMA (10 nM).

Assay of Phagocytosis. Phagocytic activity was quantitated by measuring the uptake of fluorescent microspheres (Fluoresbrite carboxylate microspheres, 1.75 μm diameter; Polysciences), as described by Blair *et al.* (7).

Analysis and Assay of PKC. PKCs in the soluble and particulate membrane fractions were separated by centrifugation after disruption of the cells, and the subspecies were analyzed by hydroxyapatite column chromatography under the conditions specified earlier (8). The enzyme was assayed by measuring incorporation of radioactive phosphate of [γ-^{32}P]ATP into a synthetic oligopeptide, MBP-(4–14) (myelin basic protein, residues 4–14), which serves as a specific substrate for PKC, as described (9).

RESULTS

Cell Differentiation by PMA and 1,2-DiC_8. HL-60 cells (5×10^5 cells per ml) were treated with a single dose of either PMA (10 nM) or DL-1,2-DiC_8 (100 μM) and incubated for 48 hr at 37°C. The patterns of CD11b expression, a marker of cell differentiation, in these cells are given in Fig. 1. Although a single dose of PMA induced this receptor expression, DL-1,2-DiC_8 was apparently inactive under the same conditions. The ability of this membrane-permeant DAG to induce cell differentiation, however, was dependent on the cell density as well as on the DL-1,2-DiC_8 concentration in the incubation medium.

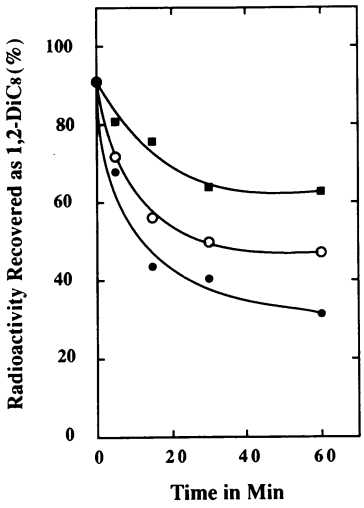

FIG. 2. Time course of 1,2-[^{14}C]DiC_8 metabolism in HL-60 cells at different densities. 1,2-[^{14}C]DiC_8 (final concentration, 100 μM) was added to 0.3 ml of cell suspensions (10^5–10^7 cells per ml). At the time indicated, lipids were extracted, and the remaining 1,2-DiC_8 was quantitated as described. ●, 1×10^7 cells per ml; ○, 1×10^6 cells per ml; ■, 1×10^5 cells per ml.

Metabolic Rate of 1,2-DiC_8. HL-60 cells appeared to take up and metabolize DL-1,2-DiC_8 very rapidly. Analysis of the metabolites on TLC suggested that this DAG was mostly hydrolyzed to produce octanoic acid and degraded further to smaller molecules rather than converted to the corresponding phosphatidic acid, 1,2-dioctanoylglyceryl-3-phosphate. The result given in Fig. 2 shows that, with a fixed amount of DL-1,2-DiC_8 (100 μM), the rate of disappearance of this DAG from the incubation medium was a function of cell density and was very fast at higher cell densities.

Availability of DAG for Cell Differentiation. Another set of experiments was performed, therefore, to examine the relationship between CD11b expression and the availability of DAG to the cell by changing the cell density and the dose of 1,2-DiC_8 in the incubation medium. The results obtained are summarized in Fig. 3. The extent of receptor expression appeared to be proportional to the duration of exposure of the cells to 1,2-DiC_8. At a low cell density (5×10^4 cells per ml),

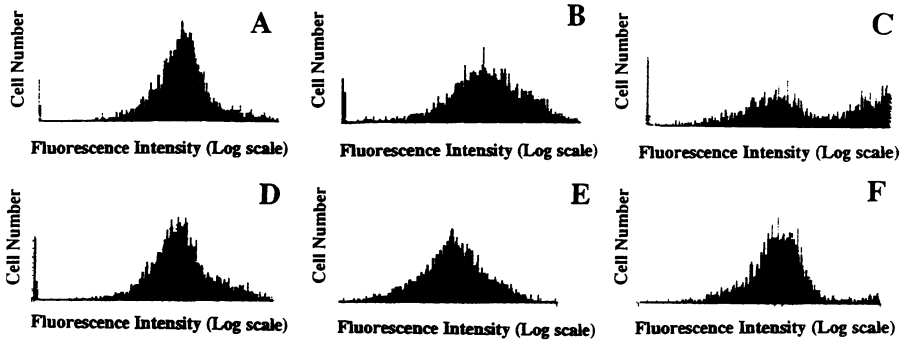

FIG. 3. CD11b expression in HL-60 cells treated with a single dose of 1,2-DiC_8. HL-60 cells (5×10^4 cells per ml or 5×10^5 cells per ml) were treated with a single dose of 1,2-DiC_8 (25–100 μM). After 48 hr, the expression of CD11b was quantitated as described. (*A*) 5×10^4 cells per ml, 25 μM 1,2-DiC_8. (*B*) 5×10^4 cells per ml, 50 μM 1,2-DiC_8. (*C*) 5×10^4 cells per ml, 100 μM 1,2-DiC_8. (*D*) 5×10^5 cells per ml, 25 μM 1,2-DiC_8. (*E*) 5×10^5 cells per ml, 50 μM 1,2-DiC_8. (*F*) 5×10^5 cells per ml, 100 μM 1,2-DiC_8.

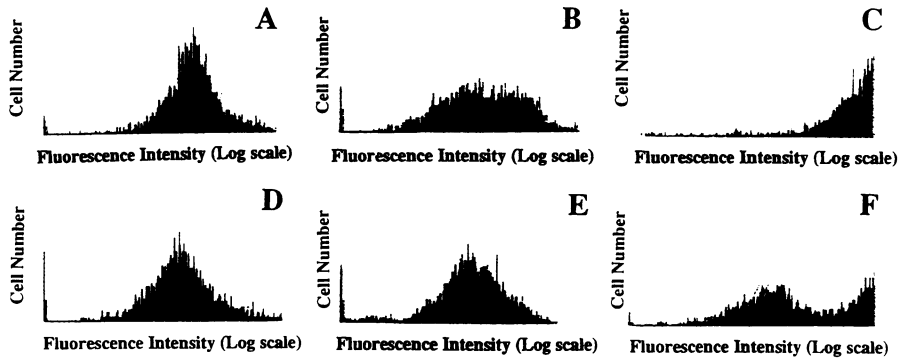

FIG. 4. CD11b expression in HL-60 cells treated with repeated doses of 1,2-DiC_8. HL-60 cells (5×10^4 cells per ml or 5×10^5 cells per ml) were treated three times with 1,2-DiC_8 (25–100 μM) at 6-hr intervals. Forty-eight hours after the first treatment, expression of CD11b was quantitated as described. (*A*) 5×10^4 cells per ml, 25 μM 1,2-DiC_8. (*B*) 5×10^4 cells per ml, 50 μM 1,2-DiC_8. (*C*) 5×10^4 cells per ml, 100 μM 1,2-DiC_8. (*D*) 5×10^5 cells per ml, 25 μM 1,2-DiC_8. (*E*) 5×10^5 cells per ml, 50 μM 1,2-DiC_8. (*F*) 5×10^5 cells per ml, 100 μM 1,2-DiC_8.

a small dose of DL-1,2-DiC_8 (25 and 50 μM) was unable to induce the cells to express the receptor (Fig. 3 *A* and *B*), whereas a large dose of the DAG (100 μM) could induce the cells to differentiate (Fig. 3*C*). In contrast, at a high cell density (5×10^5 cells per ml) 1,2-DiC_8 was unable to induce cell differentiation under the same conditions (Fig. 3 *D–F*).

Fig. 4 shows the results of repeated additions of 1,2-DiC_8 to the cell suspension at a density where cells were unable to express the receptor with a single dose of 1,2-DiC_8. It is clear that repeated doses of the DAG at 6-hr intervals greatly enhanced cell differentiation. In spite of the increased expression of CD11b, Table 1 shows that the phagocytic activity was induced slightly in the cells treated with 1,2-DiC_8, while a significant induction of this activity was observed in the cells treated with PMA.

PKC Subspecies During Differentiation by PMA. HL-60 cells contain the α- and β-subspecies of PKC and, additionally, a structurally unidentified subspecies as described (9). The addition of PMA to the cell suspension caused rapid translocation and a subsequent decrease in enzyme activity as shown in Fig. 5. After 15 min, both the β-subspecies and a structurally unknown subspecies were almost totally depleted from the cells. After 4 hr, these two subspecies gradually reappeared in association with the membrane fraction. In contrast, the α-subspecies was translocated to membranes relatively slowly and never depleted completely during the entire period of time examined, although the activity of this subspecies once decreased to $\approx$60% of the resting cell level. After 48 hr, all PKC subspecies were restored but associated with the membrane fraction, suggesting that these PKC subspecies were all still active. Enzymologically, the characteristics of the subspecies that reappeared in the membrane fraction were indistinguishable from those of the enzymes originally present in the soluble fraction in the resting cells. The relative activities of these PKC subspecies during PMA treatment are quantitatively given in Fig. 6.

Table 1. Differentiation of HL-60 cells treated with 1,2-DiC_8 or PMA

	CD11b expression, % positive cells	Phagocytic activity, % cells containing fluorescent microspheres
Control	8.83	3.56
DiC_8 (100 μM)	41.23	17.50
PMA (10 nM)	81.66	62.46

HL-60 cells (5×10^4 cells per ml) were treated as indicated. After 48 hr, the expression of CD11b was quantitated as described. The cells were incubated with 2×10^7 fluorescent microspheres per ml during the last 6 hr of incubation and phagocytic activity was quantitated as described.

PKC During Differentiation by 1,2-DiC_8. In contrast to PMA action, 1,2-DiC_8 induced neither translocation nor depletion of the PKC subspecies even when added repeatedly. A typical example of such experiments is shown in Fig. 7. In this experiment, 1,2-DiC_8 (100 μM) was added repeatedly at 15-min intervals to the cell suspension (1×10^7 cells per ml). The distribution of PKC between the soluble and membrane fractions remained unchanged within some experimental error, and no depletion of enzymatic activity was observed. On the other hand, when the cells were treated with PMA, all PKC subspecies were translocated to the membrane fraction, and nearly half of the total activity was depleted after 1 hr as described above.

DISCUSSION

Activation of PKC has been postulated to play key roles in physiological cellular responses to a variety of signals and appears to be essential for transmembrane control of cellular functions, such as release reaction and exocytosis, receptor down-regulation, and cross-talks among various signaling pathways. Recent evidence obtained with several cell systems such as activation of T lymphocytes (5, 10) and differentiation of leukemic cell line U-937 cells (11) suggests that sustained activation of PKC is needed for long-term cellular responses such as cell proliferation and differentiation. The present studies also support the notion that sustained activation of PKC for at least several hours is necessary for gene expression related to HL-60 cell differentiation. DAG added to intact cells and possibly DAG endogenously produced after stimulation by extracellular signals are metabolized very rapidly. In fact, the formation of DAG from inositol phospholipids by either a receptor-mediated or a voltage-dependent mechanism is generally transient, and the DAG thus produced in membranes is normally metabolized rapidly by DAG lipase or is converted to phosphatidic acid by DAG kinase. It has been recently shown that DAG is gradually increased at a relatively later phase in cellular responses and that this DAG is produced from signal-induced hydrolysis of phosphatidylcholine (12, 13). Obviously, the level of DAG in membranes depends on the balance of its formation and degradation. The precise biochemical mechanism to maintain the biologically active DAG at a level sufficient to prolong PKC activation remains unclear. It has been recently found that lysophosphatidylcholine acts as an additional second messenger for long-term cellular responses such as T-lymphocyte activation (14). The interactions among several second messengers such as lysophosphatidylcholine, unsaturated fatty acids, and DAG remain to be investigated.

The membrane-permeant DAG used for the present studies is a mixture of DL-isomers, 1,2-*sn*- and 2,3-*sn*-DiC_8. The

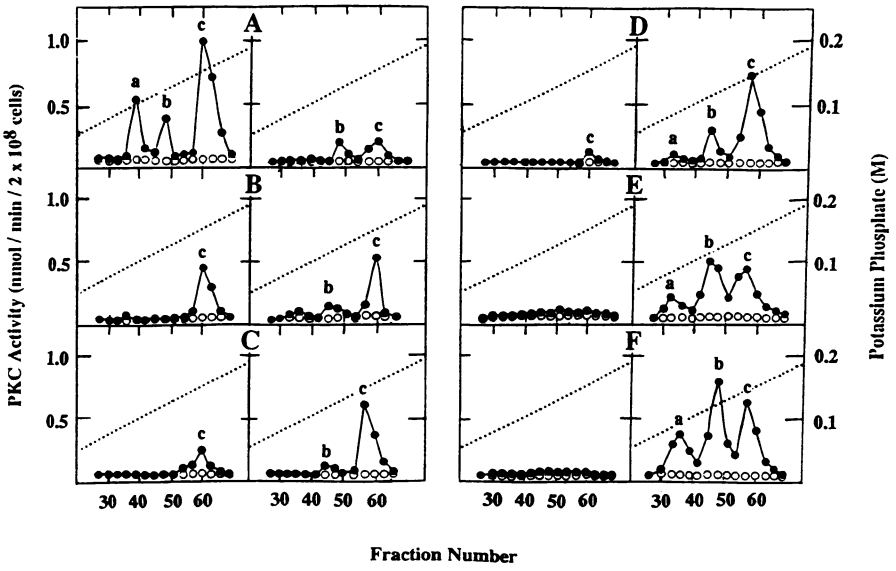

FIG. 5. PKC subspecies in soluble and membrane fractions of HL-60 cells after PMA treatment. HL-60 cells (2×10^8 cells) were treated with 10 nM PMA. At the time indicated, the cells were collected and homogenized. The PKC subspecies in the soluble and membrane fractions were separated as described. ●, PKC activity in the presence of phosphatidylserine, diolein, and calcium; ○, PKC activity in the presence of EGTA instead of phosphatidylserine, diolein, and calcium. Dotted lines indicate the concentrations of potassium phosphate. Peaks: a, the β-subspecies; b, a structurally unknown subspecies; c, the α-subspecies. Left columns, soluble fraction; right columns, membrane fraction. Time after PMA treatment: *A*, Time 0; *B*, 15 min; *C*, 1 hr; *D*, 4 hr; *E*, 24 hr; *F*, 48 hr.

biologically active form of DiC_8 for the activation of PKC should have the 1,2-*sn*-configuration (15–17). Nevertheless, the DL-forms of 1,2-DiC_8 added to the cells were degraded rapidly. It is possible that the degradation of DL-1,2-DiC_8 is a result of the action of nonspecific esterases rather than a stereospecific DAG lipase.

The results presented above seem to indicate that, although PMA frequently depletes PKC from the cell, such down-regulation of the enzyme may not be directly related to the differentiation of HL-60 cells to macrophages. Rather, it is likely that the depletion of PKC from the cell appears to represent an extreme case of PKC activation, because the PMA-induced activation of PKC may be an irreversible process. PMA has been shown indeed to cause irreversible insertion of PKC into the membrane, and there appear to be several states of the active form of PKC, although the precise mechanism of this lipid–PKC interaction remains largely unknown (18). With natural signals and also with membrane-permeant DAG, however, irreversible association of PKC to membranes was not normally detectable, although some loosely bound, digitonin-nonreleasable form of PKC was produced transiently (10). It is worth noting that, under the conditions in which HL-60 cells undergo terminal differentiation, complete depletion of the α-subspecies of PKC was never observed as described above. Presumably, the sustained and continuous activation of PKC, most likely that of the α-subspecies, is a prerequisite essential to cell differen-

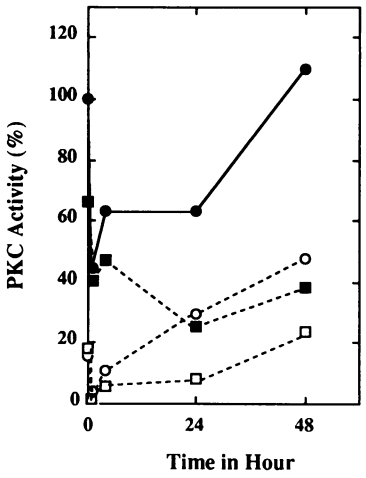

FIG. 6. Relative activities of PKC subspecies in HL-60 cells after PMA treatment. HL-60 cells were treated, and the PKC subspecies in soluble and membrane fractions were separated as shown in Fig. 5. Relative ratios of the PKC subspecies recovered in the soluble and membrane fractions are plotted. ●, Total PKC activity; ■, α-PKC activity; ○, PKC activity of the structurally unknown subspecies in hydroxyapatite column chromatography; □, β-PKC activity.

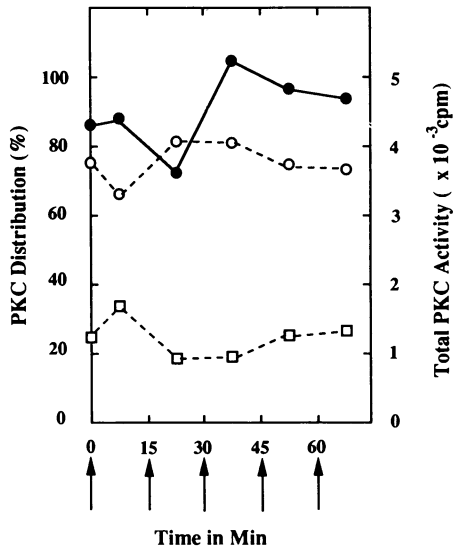

FIG. 7. PKC activity in soluble and membrane fractions in HL-60 cells treated with repeated doses of 1,2-DiC_8. HL-60 cells (1×10^7 cells per ml) were treated with repeated doses of 1,2-DiC_8 (100 μM) at 15-min intervals. At the time indicated, the cells were collected and homogenized. PKCs in the soluble and membrane fractions were assayed as described. Arrows indicate the time points when 1,2-DiC_8 (100 μM) was added. ●, Total PKC activity (cpm); ○, PKC activity in soluble fraction (%); □, PKC activity in membrane fraction (%).

tiation, eventually leading to the differentiation of HL-60 cells to macrophages. It is noted, however, that under the conditions in which CD11b was expressed significantly, multiple doses of the membrane-permeant DAG did not fully reproduce the action of PMA to induce phagocytic activity. It still remains unclear whether the apparent difference between PMA and DAG actions is due to differential activation of several PKC subspecies or to an additional biological action of this phorbol ester.

The data are taken in part from the dissertation that will be submitted by H.A. to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Sciences. We are indebted to Dr. E. Tatsumi for allowing us to use the flow cytometer in the Department of Hematology (Kobe University School of Medicine). The skillful secretarial assistance of Mrs. S. Nishiyama, Miss Y. Kimura, and Miss Y. Yamaguchi is cordially acknowledged. This work was supported in part by research grants from the Special Research Fund of the Ministry of Education, Science and Culture (Japan); the Muscular Dystrophy Association (United States); the Juvenile Diabetes Foundation International (United States); the Yamanouchi Foundation for Research on Metabolic Disorders; the Sankyo Foundation of Life Science; Merck Sharp & Dohme Research Laboratories; the Biotechnology Laboratories of Takeda Chemical Industries; and the New Lead Research Laboratories of Sankyo Company.

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