# Differential regulation of protein kinase C subspecies in human promyelocytic leukemia cell line HL-60 during differentiation induced by retinoic acid.

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# DIFFERENTIAL REGULATION OF PROTEIN KINASE C SUBSPECIES IN HUMAN PROMYELOCYTIC LEUKEMIA CELL LINE HL-60 DURING DIFFERENTIATION INDUCED BY RETINOIC ACID

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## INDEXING WORDS

protein kinase C; HL-60 cells; retinoic acid; differentiation

## SYNOPSIS

Protein kinase C (PKC) is a multifunctional protein-Ser/Thr kinase and is generally accepted to be involved in a wide variety of cellular signal transduction. Recent biochemical fractionation of PKC as well as sequence analysis of its cDNA clone has revealed the existence of multiple subspecies of this enzyme with obvious tissue- and cell-specific expression. The present study is concerned with the differential regulation of expression of PKC subspecies of human promyelocytic leukemia cell line HL-60 during differentiation toward granulocyte-like cells induced by retinoic acid. PKC in HL-60 cells is resolved into three distinct sub-

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Abbreviation used are; EGTA, ethylene glycol bis(β-aminoethylether)N,N'-tetraacetic acid; SDS, sodium dodecyl sulfate; PKC, protein kinase C; DO, diolein; PS, phosphatidylserine; RA, retinoic acid; DMSO, dimethyl sulfoxide; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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species (peak *a*, *b*, and c) by the hydroxyapatite column chromatography. Biochemical and immunochemical analyses revealed that peak *a* and c enzymes were identified with the type II (βII) and type III (α) PKC subspecies previously identified and characterized from brain. On the other hand, peak *b* enzyme was separable from type I, II and III PKC, and is suggested to be unidentified PKC subspecies possessing subtly different characters from others. Treatment of HL-60 cells with 1 μM retinoic acid caused the decrease in peak *b* activity within 24 h, while peak *a* activity was increased and peak c activity was slightly decreased within 48 h. The elution profile of PKC subspecies in human peripheral neutrophils resembled that obtained with HL-60 cells treated with RA. The results imply that activities of PKC subspcies in HL-60 cells may be distinctly regulated by retinoic acid treatment prior to the achievement of cell differentiation.

## INTRODUCTION

The physiological importance of protein kinase C (PKC) activation is generally accepted and well documented (for a review see Ref. 21). Although once PKC was thought to be a single entity, recent molecular cloning analysis has revealed the enzyme to exist as a family of multiple subspecies (for reviews see Refs. 12 and 22). Initially, four cDNA clones α, βI, βII and γ, which are highly conserved among various mammalian species, were isolated. PKC from the brain tissue is normally resolved into three subfractions, type I, II and III, upon the hydroxyapatite column chromatography, which correspond to the enzymes encoded by γ-, β(βI plus βII)- and α-cDNA clone, respectively.[10,13] Recently, three additional cDNA clones of PKC, δ, ε and ξ have been isolated from rat brain cDNA library.[23,24]

The human promyelocytic leukemia cell line HL-60 can be induced to differentiate terminally into cells with many of the characteristics of mature granulocytes or macrophages by the addition of the appropriate chemicals.[3,4,20] Thus, this cell line has been proposed to porvide a model system for studying the cellular and molecular events involved in the proliferation and differentiation. RA induces differentiation along the myeloid pathway while the phorbol ester such as TPA leads to transformation into macrophages.[2,26] PKC appears to be essential for dif-

ferentiation by TPA as a cellular receptor for the TPA through catalyzing portein phosphorylations.[19,31] On the other hand, RA does not seem to be linked directly to PKC, rather RA is shown to increase the cellular PKC activity prior to alteration of cell growth and differentiation.[5,34] By using combination of biochemical and immunochemical procedure, the present paper will describe the separation and identification of PKC subspecies expressed in control and RA-treated, differentiated HL-60 cells.

## MATERIALS AND METHODS

### *Materials*

Peptides used in this study were synthesized using Merrifield procedure[7] on an Applied Biosystems Model 430A peptide synthesizer followed by purification with gel filtration and reverse-phase column chromatography. PS and DO were purchased from Serdary Research Laboratories. RA (type XX), arachidonic acid, TPA and [$\gamma$-$^{32}$P]ATP were obtained from Sigma, Nu-Check-Prep, chemicals for Cancer Research and Du Pont-New England Nuclear, respectively. Packed columns, TSK DEAE-5PW column (0.75 x 7.5 cm), type C KB hydroxyapatite column (0.78 x 10 cm) and Superose 12 HR 10/30 column were purchased from Toso, Koken and Pharmacia-LKB Biotechnology, respectively. Rat brain PKC type I, II and III were prepared as described.[28] Human peripheral neutrophils were prepared by the Hypaque-Ficoll method.[6]

### *Cell culture*

HL-60 cells were grown at a cell density between 0.5-2.0 x $10^6$ cells/ml as suspension in RPMI 1640 media (Flow) supplemented with 5% fetal bovine serum (GIBCO) and 2 mM glutamate at 37C in a humidified 5% $CO_2$ atmosphere. Cells in their exponential growth phase were used. The basal PKC activity was not altered by cell density over the range described above. The cell differentiation was initiated by supplementing the media with 1 $\mu$M RA. RA was solublized in DMSO. The final concentration of DMSO in the media was less than 0.05%, and it did not affect the differentiation process.

*Separation and assay of PKC subspecies*

All operations were performed at 0-4C. HL-60 cells (2 x $10^8$ cells) were suspended in 4 ml of 20 mM Tris/HCl, pH 7.5, containing 0.25 M sucrose, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.4 mM leupeptin. The cells were lysed by sonication using three 15-s bursts, and centrifuged at 100,000 x g for 30 min. The supernatant from this centrifugation was designated "cytoplasmic fraction". The pellet was sonicated in 2 ml of same buffer containing 1% (V/V) Triton X-100 and centrifuged as above. The supernatant from this centrifugation designated "particulate fraction". These crude fractions were separately applied to a DEAE-5PW column, which was connected to an FPLC system (Pharmacia LKB Biotechnology) and equilibrated beforehand with Buffer A (20 mM Tris/HCl, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 2-mercaptoethanol). After washing the column with 20 ml Buffer A, PKC was eluted by increasing the concentration of NaCl linearly from 0 to 0.3 M in 20 ml Buffer A and fractions of 0.5 ml each were collected. The enzyme fraction was applied to a packed hydroxyapatite column, which was connected to an FPLC system and equilibrated beforehand with Buffer B (20 mM potassium phosphate, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10% (V/V) glycerol and 10 mM 2-mercaptoethanol). The column was washed with 28 ml Buffer B, and then, the PKC subspecies were eluted by application of a linear concentration gradient of potasium phosphate (20 to 200 mM) in 84 ml of Buffer B, at a flow rate of 0.4 ml/min. Fractions of 1 ml each were collected. Using this chromatography system, peak *a*, *b* and c enzymes were eluted at approx. 85, 110 and 135 mM potassium phosphate, respectively. The enzyme fractions were each dialyzed overnight against 20 mM Tris/HCl, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10% (V/V) glycerol, 10 mM 2-mercaptoethanol and 0.02% Triton X-100, and stored at -80C. PKC was assayed with a PKC-specific synthetic peptide substrate, $MBP_{4-14}$ (QKRPSQRSKYL), corresponding to the major phosphorylation site of myelin basic protein, under the condition described previously.[33)]

*Antibodies*

The rabbit polyclonal antibodies, designated CKpC1β-a, CKpV3α-a, CKpV5βI-a, CKpV5βII-a and CKpV3γ-a, were prepared

against the sequence-specific oligopeptides FARKGALRQKNVHEVKNHKF type II ( β ) PKC, residue 20-39 , LGPAGNKVISPSEDR type III ( α ) PKC, residue 310-324 , SYTNPEFVINV TYPE II (βI) PKC, residue 661-671 , SFVNSEFLKPEVKS type II (βII) PKC, residue 660-673 and SPIPSPSPSPTDSK type I ( γ ) PKC, residue 322-355, repectively, and were purified by column chromatography on goat anti-rabbit IgG sepharose.[15]

*Other procedures*

Electrophoresis of PKC followed by immunoblot analysis was performed as described[16] except that rabbit polyclonal antibodies and biotinylated anti-rabbit IgG were used. Lipids were dispersed in 20 mM Tris/HCl, pH 7.5, by sonication and employed as described previously.[14] $^{32}P$ was determined by counting of Cerenkov radiation.

## RESULTS

Soluble cytoplasmic fraction of cultured HL-60 cells was subjected to a DEAE-5PW column. PKC activity of the fractions from the column was assayed with a synthetic peptide, $MBP_{4-14}$, as substrate in the presence of $Ca^{2+}$, PS and DO or in the presence of EGTA instead of them (Fig. 1). A major peak with some shoulder of PKC was eluted from column at about 0.12 M - 0.15 M NaCl. When Triton X-100-extracted particulate fraction was subjected to the DEAE-5PW column chromatography under the same condition, no significant amount of PKC activity was demonstrated. Fractions 15

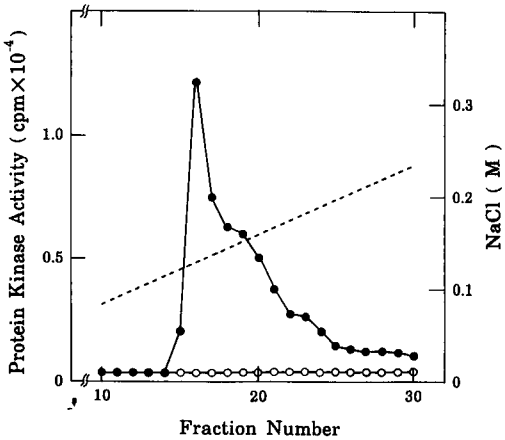


Fig. 1
DEAE-5PW column chromatography of PKC extracted from HL-60 cells. Cytoplsmic fraction of HL-60 cells (2 x $10^8$ cells) was prepared and employed for DEAE-5PW column chromatography under the condition described in "MATERIALS AND METHODS". An 8-μl aliquot of each fraction was assayed for PKC activity with $MBP_{4-14}$ as substrate. PKC activity in the presence of 0.3 mM $CaCl_2$, 8 μg/ml PS and 0.8 μg/ml DO (●) or in the presence of 0.5 mM EGTA instead of $CaCl_2$, PS and DO (○). (---), NaCl concentrations.

through 22 of DEAE-5PW column of cytoplasmic fraction were collected and applied to the hydroxyapatite column chromatography using FPLC system equipped with a packed column. Three peaks of PKC subspecies, peak *a*, *b* and c appeared (Fig. 2A). Peak *a* and c enzymes were eluted at the position corresponding to the rat brain type II and type III PKC, respectively, (Fig. 2A and D). When these three enzymes were separately subjected to gel filtration on a Superose 12 HR column using FPLC system, all enzyme showed same molecular weight of about 80 kDa (data not shown).

Immunoblot analysis following SDS/polyacrylamide gel electrophoresis indicated that both peak *a* and *b* enzymes reacted with the antibody, CKpCl -a, and the immunoreactive enzymes showed an approximate molecular weight of 82 kDa (Fig. 3A). This antibody reacted equally with the brain type I, II and III PKC.[28] The sequence which antibody, CKpClβ-a was raised against is not found in δ-, ε- and ξ-PKC.[24] Peak *a* enzyme reacted with the antibody,

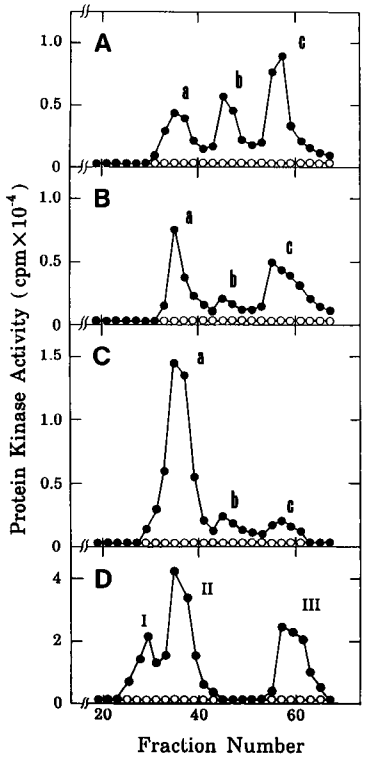


Fig. 2
Hydroxyapatite column chromatography of PKC. Pooled PKC preparation of DEAE-5PW column fraction was subjected to the hydroxyapatite column chromatography as described under "MATERIALS AND METHODS". An 8-μl aliquot of each fraction was assayed for PKC activity with $MBP_{4-14}$ as substrate. A, B, C and D; cytoplasmic fractions of undifferentiated and differentiated (4 days RA-treatment) HL-60 cells, peripheral neutrophils and rat brain, respectively. PKC activity in the presence of 0.3 mM $CaCl_2$, 8 μg/ml PS and 0.8 μg/ml DO (●) or in the presence of 0.5 mM EGTA instead of $CaCl_2$, PS and DO (○). The letters a, b, c, I, II and III designate the respective PKC fractions.

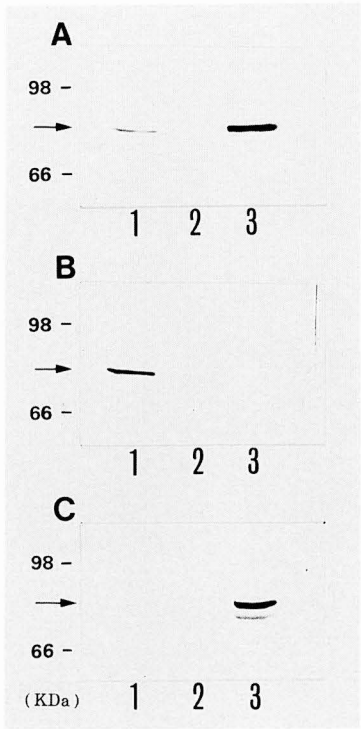


Fig. 3
Immunoblot abnalysis of PKC fraction from HL-60 cells. PKC fractions of the hydroxyapatite column chromatography were pooled and dialyzed against 0.5 mM EGTA. After freeze-drying, the samples were subjected to immunoblot analysis as described under "MATERIALS AND METHODS". A, B and C; immunoblot analysis with polyclonal antibodies, CKpCIβ-a, CKpV5βII-a and CKpV3α-a, respectively. Lanes 1, 2 and 3; peak *a*, *b* and c PKC from HL-60 cells, respectively. Arrows indicate the position of PKC. The gel was calibrated with the following standards: myosin, 205 kDa; β-glactosidase, 116 kDa; phosphorylase *b*, 98 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa.

CKpV5βII-a, which was raised against a sequence uniquely presented in the βII-PKC (Fig. 3B). Peak c enzyme was recognized by the antibody, CKpV3α-a, which was raised against a sequence uniquely expressed in α-PKC (Fig. 3C). On the other hand, the peak *a*, *b* and c enzymes all did not react with the antibodies which were raised against unique sequences of βI- and γ-PKC (data not shown). Thus, peak *a* and c enzymes were identified as the enzymes corresponding to the rat brain βII- and α-PKC, respectively. Peak *b* enzyme did not react with any antibodies against α-, βI-, βII- and γ-PKC. Whereas δ-, ε- and ζ-PKC have been shown to be phospholipid-dependent but $Ca^{2+}$-independent enzymes,[23,25] peak *b* required $Ca^{2+}$ for its activity (see below). These suggests that peak *b* enzyme is unidentified PKC subspecies.

Enzymological studies with $MBP_{4-14}$ as a phosphate acceptor revealed that three enzymes all possessed PKC natures but peak *b* enzyme showed distinctly different properties. As described for rat brain enzymes,[28] all peaks of enzyme were activated by DO or TPA to a variable extent in the presence of both $Ca^{2+}$ and PS (Fig. 4). At high concentrations of $Ca^{2+}$, PS alone caused marked activation of peak *a* and c enzymes giving almost full enzymatic activity. Whereas, peak *b* enzyme responded well to DO or TPA in

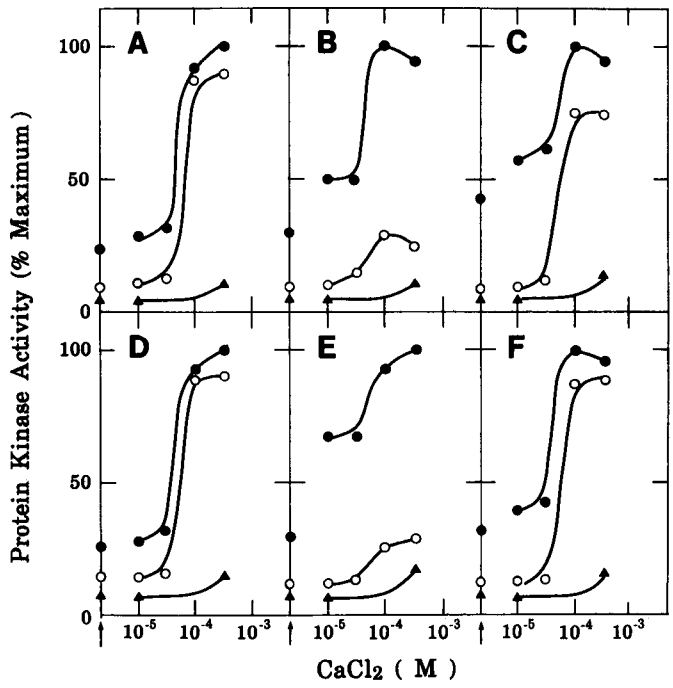


Fig. 4
Activation of PKC fractions by PS and DO or TPA at various concentrations of $CaCl_2$. The fractions of peak *a*, *b* and c were assayed at various concentrations of $CaCl_2$ as described under "MATERIALS AND METHODS". An 8-μl aliquot of each fraction was assayed for PKC activity with $MBP_{4-14}$ as substrate. A and D, peak *a*; B and E, peak *b*; C and F, peak c. A, B and C: with DO (0.8 μg/ml); D, E and F: with TPA (10 ng/ml). PKC activity in the presence of PS (8 μg/ml) plus either DO or TPA ( ● ), in the presence of PS (8 μg/ml) alone ( ○ ) and in the presence of DO or TPA alone ( ▲ ). Where indicated with arrows, EGTA (0,5 mM final concentration) was added instead of $CaCl_2$. Results were normalized to the maximum activity obtained in the presence of $CaCl_2$, PS and either DO or TPA.

the presence of a wide range of $Ca^{2+}$, and still needed DO or TPA for full activity in the presence of 0.3 mM $Ca^{2+}$. Several other kinetics of peak *a*, *b* and c enzymes were examined. Half maximum concentrations of DO for the activation of peak *a*, *b* and c enzymes were about 0.15 μg/ml, 0.25 μg/ml and 0.1 μg/ml, respectively (Fig. 5A). As described presviously with rat brain PKC,[28] peak *a*, *b* and c enzymes were activated by arachidonic acid in the rpesence of $Ca^{2+}$. Nevertheless, arachidonic acid was much less effective toward peak *b* enzyme (Fig. 5B). House and Kemp[9] has first described that the peptide, $PKC_{19-31}$ (RFARKGALRQKNV), which corresponds to a pseudosubstrate region of α-, β- and γ-PKC

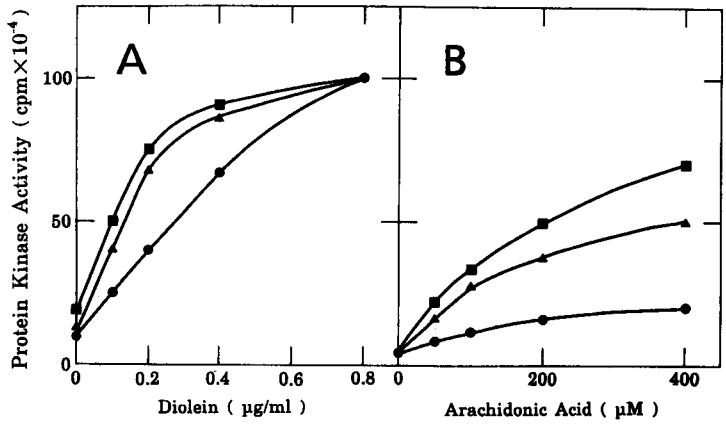


Fig. 5

Activation of PKC subspecies by DO and arachidonic acid. A, Activation of PKC fractions by DO. Peak *a* ( ▲ ), *b* ( ● ) and c ( ■ ) enzymes were assayed in the presence of PS (2 μg/ml), $CaCl_2$ (3 μM) and various concentrations of diolein as indicated. Results were normalized to the activity obtained by the addition of DO (0.8 μg/ml). B, activation of PKC fractions by arachidonic acid. Peak *a* ( ▲ ), *b* ( ● ) and c ( ■ ) enzymes were assayed in the presence of 0.3 mM $CaCl_2$ with various concentrations of arachidonic acid. Neither PS nor diolein was added. Results were normalized to the activity obtained in the presence of PS (8 μg/ml), DO (0.8 μg /ml) and $CaCl_2$ (0.3 mM).

subspecies, inhibits PKC activity at micromolar concentrations. Peak *a* and c enzyme were equally inhibited by the peptide as brain enzyme (Fig. 6). Whereas, peak *b* enzyme was much less sensitive to the peptide.

RA has been repeatedly shown to induce the differentiation of HL-60 cells along the myeloid pathway.[2)] In this study, the relative velocity of cell growth was decreased by about 50% and more than 30% of HL-60 cells became to show a granulocyte-like segmented nucleus after cultured for 5 days in the medium containing 1 μM RA (data not shown). PKC in the HL-60 cells treated for 4 days with RA was extracted and subjected to the DEAE-5PW column chromatography followed by the hydroxyapatite column chromatography under the same condition for the analysis of the control cells. PKC activity was again recovered in soluble fraction and peak *a*, *b* and *c* enzyme were separated (Fig. 2B) It is worth noting that the elution profile of PKC subspecies was quite different from that obtained with the control cells. Peak *a* enzyme (βII-PKC) was increased about 2-fold. Peak c enzyme corresponding to α-PKC was slightly decreased. Peak *b* enzyme was extensively decreased. Using a preparation of human peripheral neutrophils, PKC fractions were resolved into three distinct subspecies under the same

condition as that of HL-60 cells by the hydroxyapatite column chromatography. The three peaks were eluted at the position corresponding to the HL-60 cells PKC peak *a*, *b* and c (Fig. 2C). The pattern of elution of the three peaks resembled that of HL-60 cells treated with RA. Finally, relative alterations in the activities of cytoplasmic PKC subspecies with time following the addition of RA are shown in Fig. 7. There were marked alterations in the activities within 48 h following RA addition to the culture medium and prior to the significant appearance of morphological change, which was observed after 5 days. Espeacially, the decrease in peak *b* enzyme was completed within 24-h RA treatment.

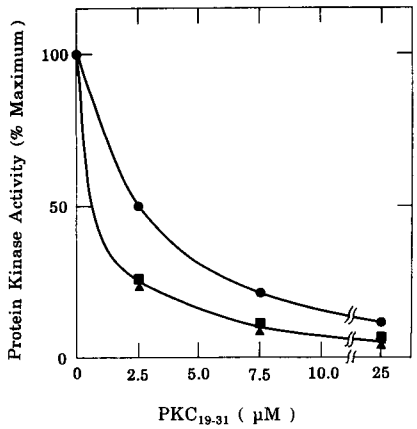


Fig. 6
Inhibition of PKC fractions by the peptide, $PKC_{19-31}$. Peak *a* (▲), *b* (●) and c (■) enzymes were assayed in the presence of 0.3 mM $CaCl_2$, 8 μg/ml PS and 0.8 μg/ml DO with various concentrations of $PKC_{19-31}$ as indicated. Results are normalized to the activity obtained in the absence of the peptide.

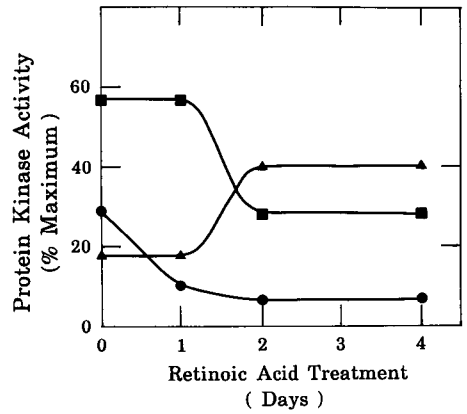


Fig. 7
Relative alteration in the activities of PKC fractions of HL-60 cells with time of RA treatment. HL-60 cells were cultured with 1 μM RA as described under "MATERIALS AND METHODS" for various days as indicated. Then, cytoplasmic PKC fractions of HL-60 cells were separated and quantitated through the DEAE-5PW and hydroxyapatite column chromatographies as described under "MATERIALS AND METHODS". (▲), (●) and (■); peak *a*, peak *b* and peak c activity, respectively. Results are normalized to the total PKC activity (peak *a* + peak *b* + peak c) found at 0 day.

## DISCUSSION

Previous reports from this laboratory[27,30] has shown the differential co-expression of several PKC subspecies among human

leukemia and lymphoma cell lines of various differentiation stages and T lymphocytes. The present biochemical and immunoblot analyses indicate that HL-60 cells and human peripheral neutrophils have type II (β) and III (α) PKC and an additional structurally unknown PKC subspecies (peak *b* enzyme). The expressions of each PKC subspecies were differentially regulated during RA treatment of the cells, which induced the differentiation of the cells to granulocytes. Type II (β) PKC was increased and type III (α) and unknown PKC (peak *b*) were decreased. Finally, the expression of PKC subspecies became to be similar to that in human peripheral neutrophils. It is worthy to note that the decrease of peak *b* enzyme was observed at first within 24 h after the addition of RA to the medium. Thus, peak *b* enzyme may have crucially important roles in cell growth and differentiation, although this enzyme was not yet demonstrated in rat tissues such as brain, liver and kidney. Makowske et al.[18] previously reported that PKC in HL-60 cells was resolved into three subfractions, which were all induced in parallel during differentiation after treatment with either RA or DMSO. Although these authors thought that the three PKC subfractions were type I, II and III PKC, the studies of our and other laboratories have shown that type I (γ) PKC is expressed only in the central nervous tissues and spinal cord.[11,17]

In contrast to the Ra-induced differentiation, exposure of HL-60 cells to TPA results in the macrophage-like differentiation, accompanying phosphorylation of several endogenous proteins.[3,19] TPA causes rapid translocation to membranes and susequent disappearance, down-regulation, of PKC. Several mutant cell lines, which do not translocate or down-regulate PKC, do not appear to differentiate to macrohphage-like cells in response to TPA.[8,29] On the other hand, 1-oleoyl-2-acetylglycerol, which activates PKC *in vivo* and *in vitro*, was reported not to induce the differentiation of HL-60 cells.[32] Sustained activation of PKC may be necessary for the induction of the differentiation of HL-60 cells. Activation of T lymphocytes was shown to require such sustained activation of PKC.[1] In summary, available evidences including the present experiment suggest that the members of the PKC family in HL-60 cells play different fucntions and are distinctly regulated during cell differentiation.

## ACKNOWLEDGMENTS

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that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid.

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