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Differential down-regulation of protein kinase C subspecies in KM3 cells

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The down-regulation of protein kinase C (PKC) subspecies in KM3 cells (a pre-B, pre-T cell line) has been examined. The PKC from KM3 cells was resolved into two subspecies, type II (mainly βII) and type III (α), upon hydroxyapatite column chromatography. Biochemical and immunocytochemical analysis revealed that, when these cells were treated with 12-*O*-tetradecanoylphorbol 13-acetate (TPA), the time course of down-regulation of the PKC subspecies was different; type II PKC was translocated and depleted from the cell more quickly than type III enzyme. The results suggest that each PKC subspecies plays a different role in the cellular response to TPA and probably to other external stimuli.

Protein kinase C; Enzyme subspecies; Down-regulation

1. INTRODUCTION

Tumor-promoting phorbol esters elicit a variety of biological responses in tissues and cultured cells (reviews [1,2]). PKC has been generally accepted as a major receptor for phorbol esters [3,4], which stimulate its rapid translocation from the cytosol to the membrane and subsequent depletion in many cell types [5,6]. At that time PKC is proteolytically activated by calpain [7–9]. Long-term stimulation by TPA often causes either differentiation or proliferation of various cell lines.

Molecular cloning studies indicate that PKC exists as a family of subspecies; four cDNA clones named α-, βI-, βII- and γ-PKC were initially found (review [10]). Recently, another group of cDNA clones designated δ-, ε- and ζ-PKC have been isolated [11]. On the other hand, rat brain PKC can be resolved by hydroxyapatite column chromatography into three fractions, types I–III [12,13]. Analysis of the PKC subspecies expressed in COS 7 cells transfected by γ-, β(βI + βII)- and α-cDNAs shows that these encode type I, II and III PKC, respectively [14]. Some cell lines, such as NIH 3T3 [15], A431 [16] and COS 7 cells [13], contain only type III (α) PKC, but a variety of other tissues [17] and cell types, including T lymphocytes [18], co-express various PKC subspecies. Such tissue- and cell-specific expression of PKC subspecies suggests that each member of the enzyme family has a different function in cell responses to external stimuli. This paper describes a biochemical and immunocytochemical analysis of the PKC subspecies and their differential mode of down-regulation in the clonal pre-B, pre-T cell line, KM3 [19].

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Abbreviations: PKC, protein kinase C; FPLC, fast protein liquid chromatography; TPA, 12-*O*-tetradecanoylphorbol 13-acetate

2. MATERIALS AND METHODS

2.1. *Materials and chemicals*

Calf thymus H1 histone was prepared as in [7]. Phosphatidylserine and 1,2-diolein were from Serdary Research Laboratories. [γ-^{32}P]ATP was purchased from Amersham. TPA was obtained from Chemicals for Cancer Research.

2.2. *Cells*

The human pre-B, pre-T leukemic cell line, KM3 [19], was kindly provided by Dr J. Minowada (Hayashibara Biochemical Laboratories, Inc.) and cultured in RPMI 1640 (Flow) sup-

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plemented with 5% fetal calf serum (Gibco), 2 mM glutamine and penicillin-streptomycin (50 U/ml and 50 μg/ml, respectively) at 37°C in 5% CO_2. In all experiments cells were used at a density of 1×10^6 cells/ml.

2.3. *Partial purification of PKC from KM3 cells*

KM3 cells (approx. 2×10^8 cells) were suspended in 4 ml of 20 mM Tris-HCl (pH 7.5) containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 100 μg/ml leupeptin. The cells were lysed by sonication using three 15-s bursts, and centrifuged at 100000 × g for 30 min. The supernatant from this centrifugation was designated 'cytoplasmic fraction'. The pellet was re-sonicated in 2 ml of the same buffer containing 1% (v/v) Triton X-100 and re-centrifuged as above. The supernatant from this centrifugation was designated 'particulate fraction'. These crude fractions were separately applied to a 4 ml DE-52 column equilibrated with 20 mM Tris-HCl (pH 7.5) containing 0.5 mM EDTA, 0.5 mM EGTA and 10 mM 2-mercaptoethanol (buffer A). After washing with 2 column volumes of buffer A, followed by 3 column volumes of buffer A containing 20 mM NaCl, PKC was eluted batchwise with 3 column volumes of buffer A containing 120 mM NaCl. The eluate was resolved by chromatography on a hydroxyapatite column connected to an FPLC system as described [14]. Using this chromatography system, type I, II and III PKCs were eluted at approx. 90, 100 and 150 mM potassium phosphate, respectively.

2.4. *Assay of PKC*

The enzyme was assayed with calf thymus H1 histone as a phosphate acceptor in the presence of phosphatidylserine (8 μg/ml), diolein (0.8 μg/ml) and 0.3 mM Ca^{2+} as in [14]. Blank incubation was performed by replacing $CaCl_2$ with 0.5 mM EGTA without addition of lipids.

2.5. *Immunocytochemical analysis of PKC subspecies in KM3 cells*

After treatment, cells were fixed in acetone for 20 min at room temperature and washed twice with phosphate-buffered saline. Aliquots of these cells (approx. 2×10^5 cells) were cytocentrifuged onto gelatin-coated glass slides. Cells on the slide were permeabilized by incubation in 0.1% Triton X-100 for 20 min at room temperature. After washing, cells were incubated overnight at 4°C with appropriate polyclonal anti-PKC antibodies containing 0.02% normal human serum. The polyclonal antibodies, designated CKpV5βI-a, CKpV5βII-a and CKpV1α-a, were prepared against the sequence-specific oligopeptides SYTNPEFVINV [type II (βI) PKC, residues 661–671], SFVNSEFLKPEVKS [type II (βII) PKC, residues 660–673] and VYPANDSTASQDVANR [type III (α) PKC, residues 4–19], respectively. These antibodies were purified by column chromatography on anti-IgG Sepharose and on the corresponding peptide coupled to Sepharose. The localization of PKC subspecies in the cells was visualized using an anti-rabbit immunoglobulin streptavidin/biotin-peroxidase immunostaining kit (Biogenex) according to the manufacturer's instructions, except that samples were incubated with biotin-conjugated anti-rabbit immunoglobulins and streptavidin at 4°C for 2 and 1 h, respectively. 3,3′-Diaminobenzidine was employed as substrate for the peroxidase.

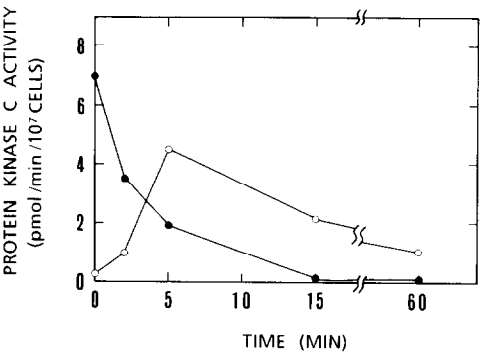

Fig.1. Time course of PKC translocation and down-regulation in KM3 cells. KM3 cells were treated with 50 nM TPA for the indicated times, and crude fractions were prepared and assayed as described in section 2. PKC activity was defined as the kinase activity measured in the presence of phospholipid and Ca^{2+} minus that detected in the absence of phospholipid and with Ca^{2+} replaced by 0.5 mM EGTA. PKC activity in (●) cytoplasmic and (○) particulate fractions.

3. RESULTS

Fig.1 shows the time course of down-regulation of PKC in KM3 cells in response to treatment with 50 nM TPA. At time 0 more than 98% of PKC activity was found in the cytoplasmic fraction. PKC activity in this fraction was decreased by approx. 50% at 2 min after treatment with TPA. At 15 min the soluble PKC activity was undetectable. Some of the PKC activity was recovered in the particulate fraction, with maximal activity at 5 min. After 60 min PKC activity remained only in the particulate fraction (approx. 15% of total PKC activity at time 0).

Immunocytochemical staining of PKC during the treatment with TPA is demonstrated in fig.2. KM3 cells are stained strongly by CKpV5βII-a (anti-βII) as well as CKpV1α-a (anti-α), but very weakly by CKpV5βI-a (anti-βI). No staining was observed with antibody against γ-PKC. The result indicates that KM3 cells express α-, βII- and a small quantity of βI-, but not γ-PKC subspecies. This conclusion was supported by immunoblotting analysis. In untreated control cells, βII- and α-PKC were both located predominantly in the cytoplasm (fig.2A,E). At 5 min after treatment with TPA (50 nM) the staining intensity of βII-PKC was markedly decreased compared with the intensity observed at time 0, while staining of α-PKC was of the same intensity as at time 0. When

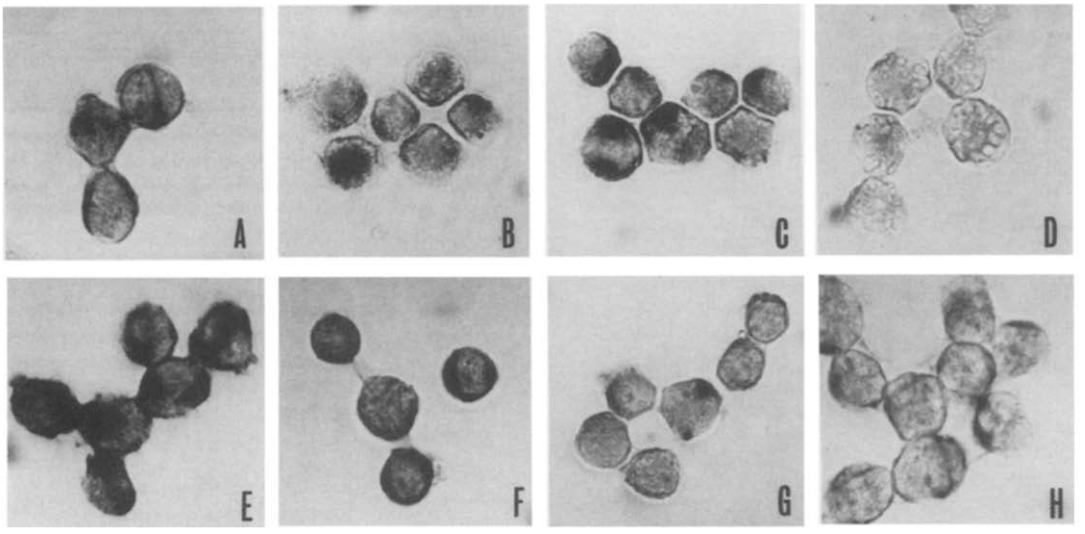

Fig.2. Time course of immunocytochemical localization of PKC subspecies in KM3 cells. KM3 cells were treated with 50 nM TPA for the indicated times. Cells were then rapidly cooled by addition of ice-cold medium, and after centrifugation, stained as described in section 2. (A–D) Staining by CKpV1α-a, (E–H) staining by CKpV5βII-a. (A,E) At time 0, (B,F) 2 min, (C,G) 5 min, (D,H) 180 min after treatment with TPA.

these cells had been treated for 3 h, the staining intensity of both α- and βII-PKC had decreased, although some granular staining was observed in the cytoplasm and perinuclear regions of some cells. βI-PKC disappeared in a manner essentially similar to that of βII-PKC, although the staining intensity was very weak. The disappearance of PKC subspecies was not due to death of the cells as judged by trypan blue exclusion.

The immunocytochemical analysis described above suggests that the time course of depletion of PKC subspecies is different. This possibility was examined further biochemically by isolating PKC subspecies from TPA-treated cells by hydroxyapatite column chromatography. Consistent with the immunological studies, two distinct enzyme fractions were eluted from the hydroxyapatite column at approx. 100 and 150 mM potassium phosphate, which corresponded to type II (β) and type III (α) PKC, respectively (fig.3). Immunoblotting analysis indicated that type II consists of mainly βII-PKC, with little, if any βI-PKC. In the control cells PKC was found in the cytoplasmic but not the particulate fraction (fig.3A,G). After TPA treatment type II and type III PKC in the cytosol were rapidly decreased. Type II was translocated to the particulate fraction (approx. 15% of the activity in the untreated cytoplasmic fraction) at 2 min (fig.3B,H). At 15 min, type II PKC had disappeared in both soluble and particulate fractions, whereas type III PKC translocation to the particulate fraction had reached the maximum level (approx. 20% of the type III PKC activity in the untreated cytoplasmic fraction) (fig.3D,J). At 3 h, a small amount of type III PKC (approx. 1%) remained in the particulate fraction (fig.3K), and at 6 h there was no detectable PKC in any fractions (fig.3F,L). These profiles are more quantitatively plotted in fig.4.

During the entire time course examined, a Ca^{2+}-, phospholipid- and diacylglycerol-independent protein kinase activity was slightly increased in the soluble fraction with the maximum value at 15 min (approx. 20% of that activity in the untreated control cells). However, definitive evidence was unavailable indicating that this activity is due to protein kinase M, a proteolytically produced catalytic fragment of PKC [7].

4. DISCUSSION

Previous studies have shown that treatment with TPA elicits translocation and down-regulation (depletion) of PKC in a variety of cell types [5,6].

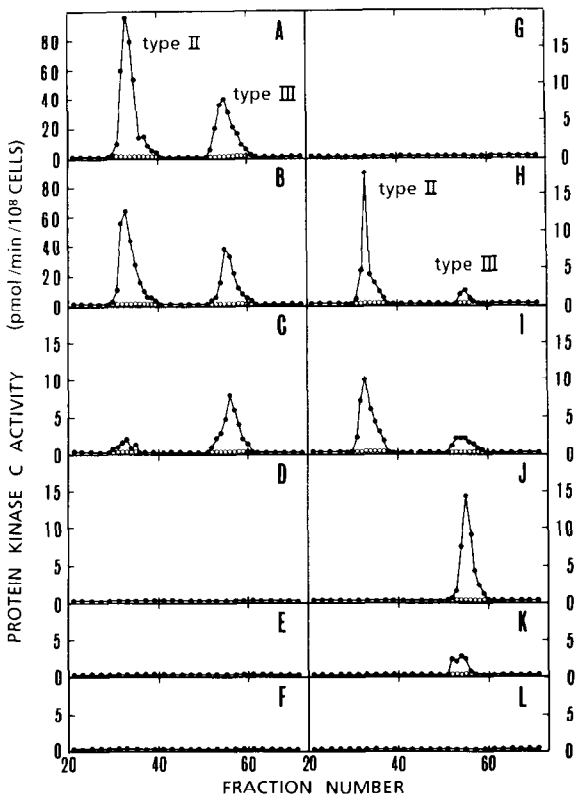

Fig.3. Translocation and down-regulation of PKC subspecies in KM3 cells. KM3 cells were treated with 50 nM TPA for the indicated times. PKC subspecies were resolved and assayed as described in section 2. Activity of PKC subspecies in (A–F) cytoplasmic and (G–K) particulate fractions. (A,G) At time 0, (B,H) 2 min, (C,I) 5 min; (D,J) 15 min, (E,K) 180 min, (F,L) 240 min after TPA treatment. PKC activity in the presence of (●) phosphatidylserine, diolein and $CaCl_2$ and of (○) EGTA instead of phosphatidylserine, diolein and $CaCl_2$.

The results presented above show that, when treated with TPA, PKC subspecies co-expressed in a single cell, KM3, are apparently translocated to the particulate fraction and subsequently depleted at different rates. This depletion of PKC molecules appears to be initiated by the action of calpain I, since this protease cleaves preferentially the active form, but not the inactive resting form of PKC [7]. Recent analysis in this laboratory indicates that the limited proteolysis of PKC takes place at a site in the third variable region, V3 [21], which connects the regulatory and protein kinase domains (Kishimoto et al., unpublished). Consistent with the observation described here, type II PKC is more susceptible to calpain I than type III PKC

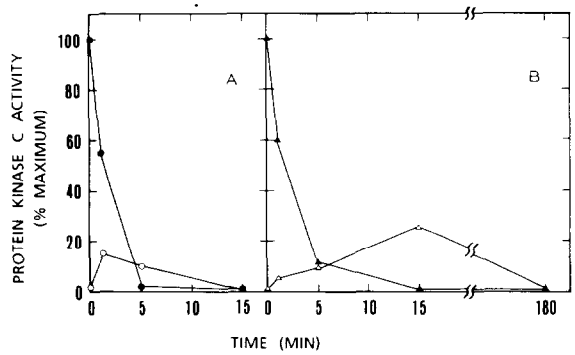

Fig.4. Time course of translocation and down-regulation of PKC subspecies in KM3 cells. KM3 cells were treated with TPA, and PKC activity in each fraction was analyzed as described in fig.3. (A) Type II, (B) type III; PKC activity of (▲,●) cytoplasmic and (△,○) particulate fractions.

under comparable conditions.

The limited proteolysis of PKC may generate a fragment of PKC of approx. 51 kDa, protein kinase M, which is fully active, in vitro, without added Ca^{2+}, phospholipid, and diacylglycerol [7]. Two alternative possibilities for the significance of this mechanism have thus far been discussed; firstly, that this proteolysis is a process to activate PKC and that the resulting fragment may play some roles in the control of cellular functions [9,22]; and secondly, that this is a process for initiating the degradation of PKC, eventually leading to its disappearance, termed down-regulation [6,23]. However, at present, no obvious evidence is available to support the first possibility. Upon treatment of KM3 cells with TPA, Ca^{2+}-, phospholipid- and diacylglycerol-independent protein kinase activity was slightly increased in the cytoplasmic fraction as noted above. However, all biochemical and immunological approaches have failed to identify this activity as protein kinase M. Rather, it is worth noting that the total protein kinase activity originally found in intact KM3 cells is rapidly depleted and disappears after treatment with TPA. It is attractive to investigate further whether the limited proteolysis by calpain I does initiate the down-regulation of various PKC molecules.

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