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LIMITED PROTEOLYSIS AND DIFFERENTIAL DOWN-REGULATION OF PROTEIN KINASE C SUBSPECIES IN KM3 CELLS

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INDEXING WORDS

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SYNOPSIS

Protein kinase C (PKC) is known to play crucial roles in signal transduction. This enzyme was originally found as an undefined protein kinase, which could be activated by limited proteolysis by a Ca^{2+}-dependent neutral protease, calpain. PKC was thought to be a single entity for many years, but recent, molecular cloning and biochemical analysis has shown that this enzyme consists of multiple subspecies with closely related structures. The present studies were undertaken to explore the susceptibility of PKC subspecies to proteolysis by calpain in KM3 cells, a pre-B, pre-T cell line. The PKC from KM3 cells was resolved into two

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Abbreviations used are: PKC, protein kinase C; PKM, protein kinase M; EGTA, ethylene glycol bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; EDTA, ethylene diamine tetraacetic acid; FPLC, fast protein liquid chromatography; TPA, 12-*O*-tetradecanoylphorbol-13-actate.

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subspecies, type II (mainly βII) and type III (α), upon hydroxyapatite column chromatography. This cell line was also shown to contain calpain I and calpain II, which show different sensitivities to Ca^{2+}. Both type II and type III PKC were hydrolysed by either calpain I or calpain II, but type II PKC was cleaved more effectively than type III. The simultaneous presence of phospholipid and diacylglycerol enhanced the cleavage of these PKC subspecies by calpain I. When these cells were treated with phorbol ester, the rates of translocation and down-regulation of these PKC subspecies were different; type II PKC was translocated and depleted from the cell more rapidly than type III enzyme. The results presented herein suggest different roles of the PKC subspecies in the cellular responses to external stimuli.

INTRODUCTION

Protein kinase C (PKC) requires Ca^{2+} and phospholipid for its activation. Diacylglycerol, the product of phosphatidylinositol hydrolysis that remains in membranes, dramatically increases the affinity of this enzyme for Ca^{2+} and thereby renders it fully active without a net increase in the Ca^{2+} concentration.[14] PKC is generally accepted as a major receptor for phorbol esters, such as TPA, which can substitute for diacylglycerol to activate PKC.[2,13] Phorbol esters stimulate rapid translocation of the enzyme from cytosol to membrane, and subsequent depletion from many cell types.[10,18] Long-term stimulation by TPA often causes either differentiation or proliferation of various cell lines.[14]

Molecular cloning studies have indicated that PKC exists as a family of multiple subspecies. Initially four cDNA clones named α, βI, βII, and γ were found.[15] On the other hand, rat brain PKC can be resolved by hydroxyapatite column chromatography into three fractions, type I, type II, and type III.[5,16] Analysis of the PKC subspecies expressed in COS 7 cells transfected by γ-, β (βI+βII)-, and α-cDNAs shows that these clones encode type I, II, and III PKC, respectively.[6] Some cell lines, such as NIH 3T3[11] contain only type III (α) PKC, but a variety of tissues , and cell types, including T lymphocytes,[20] co-express various PKC subspecies. Recently, additional members of PKC subspecies, designated δ-, ε- and ζ-PKC have been isolated.[17]

Protein kinase M (PKM), a catalytic fragment of PKC can be produced by limited proteolysis of PKC by Ca^{2+}-dependent neutral protease, calpain.[7,12,22] PKM is fully active, *in vitro*, without addition of Ca^{2+}, phospholipid, and diacylglycerol. All subspecies of PKC from rat brain were revealed to be cleaved by calpain I and calpain II from rat kidney in a certain region of each PKC molecule and the relative rates of cleavage were different from each other *in vitro*.[8]

This paper will describe biochemical analysis of the PKC subspecies, their different susceptibility to calpain, and differential down-regulation in a clonal pre-B, pre-T cell line, KM3.

MATERIALS AND METHODS

Materials and chemicals

Calf thymus H1 histone was prepared as described by Hashimoto et al.[4] Phosphatidylserine and 1,2-diolein were obtained from Serdary Research Laboratories. Phospholipid was extracted and fractionated as described.[7] [γ-^{32}P]ATP and bovine serum albumin (fatty acid free) were purchased from Amersham and Armour, respectively. TPA was obtained from Chemicals for Cancer Research.

Cells

The human pre-B, pre-T leukemic cell line, KM3,[19] was kindly provided by Dr. J. Minowada (Hayashibara Biochemical Laboratories, Inc.) The cells were cultured at 37C in 5% CO_2 in RPMI 1640 (FLOW) supplemented with 5% fetal calf serum (GIBCO), 2 mM glutamine and penicillin-streptomycin (50 units/ml and 50 μ g/ml, repectively). In all experiments cells were used at a density of 1 x 10^6 cells/ml.

Partial purification of PKC

All procedures were carried out at 0-4C. KM3 cells (approximately 2 x 10^8 cells), 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, were lysed by sonication using three 15-seconds bursts. The lysate was centrifuged at 100,000 x g for 30 min. The supernatant was

designated as "cytosol fraction." The pellet was sonicated again in 2 ml of the same buffer containing 1% (v/v) Triton X-100 and centrifuged as above. The supernatant from this centrifugation was designated as "particulate fraction." These crude fractions were separately applied to a DE-52 column (1 x 5 cm) 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 batch-wise with 3 column volumes of buffer A containing 120 mM NaCl. The eluate was applied to a packed hydroxyapatite column (0.78 x 10 cm) connected to Pharmacia FPLC system and equilibrated with 20 mM potassium phosphate buffer at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10% glycerol, and 10 mM 2-mercaptoethanol (buffer B). The column was washed with buffer B, and PKC was eluted by application of a linear concentration gradient of 84 ml of potassium phosphate at pH 7.5 (20 to 215 mM) prepared in buffer B as described.[5,16] Fractions (1 ml) were collected.

Partial purification of calpain

All procedures were carried out at 0-4C. KM3 cells (approximately 2 x 10^8 cells) suspended in 4 ml of 20 mM Tris/HCl (pH 7.5) containing 0.25 mM sucrose, 10 mM EGTA, 5 mM EDTA, and 50 mM 2-mercaptoethanol, were lysed by sonication using three 15-seconds bursts. The lysate was centrifuged at 100,000 x g for 30 min. The supernatant was applied to a DE-52 column (1 x 5 cm) equilibrated with 20 mM Tris/HCl (pH 7.5) containing 10 mM EGTA, 5 mM EDTA, and 50 mM 2-mercaptoethanol (buffer C). After the column was washed with 10 column volumes of buffer C, calpain was eluted from the column by the application of linear concentration gradient of NaCl in buffer C (0 to 500 mM NaCl) prepared in 50 ml of buffer C. Fractions (1 ml) were collected. The fractions containing calpain activity were pooled, and the enzyme was purified further by chromatography on Sephacryl S-300 column equilibrated with buffer C containing 50 mM NaCl.

Assay of protein kinase

The enzyme was assayed with calf thymus H1 histone as a phosphate acceptor in the presence or absence of 8 μg/ml phos-

phatidylserine, 0.8 μg/ml diolein, and 0.3 mM $CaCl_2$ as described previously.[16] PKM was assayed by replacing $CaCl_2$ with 0.5 mM EGTA and lipids were omitted from the reaction mixture.[7]

Assay of calpain

Calpain was assayed with ^{125}I-casein as substrate in the presence of various concentrations of $CaCl_2$ by measuring the formation of acid-soluble radioactive materials as described previously.[7] Ca^{2+} concentration of reaction mixture was calculated by the method of Bartfai.[1]

Proteolysis of PKC with calpain

PKC subspecies from KM3 cells were incubated with calpain from KM3 cells in the presence of 10 mM Tris/HCl (pH 7.5) containing 0.2 mM $CaCl_2$, 1% Tween20, 20 mM 2-mercaptoethanol, 0.2% fatty acid-free bovine serum albumin, and in the presence or absence of 8 μg/ml phospholipid and 0.8 μg/ml diolein. After incubation for 20 minutes, aliquots of the reaction mixture were removed and immediately assayed for PKM activity.

RESULTS

When the cytosol fraction of KM3 cells was applied to the hydroxyapatite column two distinct enzyme fractions were eluted at approximately 100 mM and 150 mM potassium phosphate concentration, which corresponded to type II (β) and type III (α) PKC (fig. 1). Immunoblot analysis indicated that the type II consists of mainly βII-PKC and little, if any, βI-PKC.

Chromatography of the cytosol fraction of KM3 cells on the DE-52 column produced two peaks of Ca^{2+} dependent protease activity, eluting at approximately 130 mM (peak I) and 250 mM (peak II) NaCl concentration (fig. 2). The elution profile of the two peaks from the DE-52 column is similar to that of calpain I and calpain II from other tissues.[7] After subjection to gel filtration on a Sephacryl S-300 column to remove endogenous calpain inhibitors, each peak was assayed at various concentrations of Ca^{2+}. Peak I is more sensitive to Ca^{2+} than peak II (fig. 3). The results indicate that peak I and peak II from KM3 cells correspond to calpain I and calpain II, respectively.

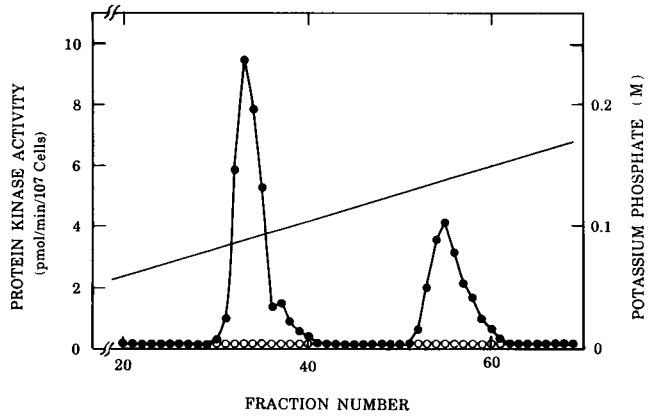

Fig. 1 Isolation of PKC subspecies from KM3 cells. The cytosol fraction from KM3 cells was subjected to DE-52 and hydroxyapatite column chromatography. The protein kinase activity was measured as described under "MATERIALS AND METHODS." (●—●), protein kinase activity in the presence of phosphatidylserine, diolein, and $CaCl_2$; (○—○), protein kinase activity in the presence of 0.5 mM EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; (——), potassium phosphate.

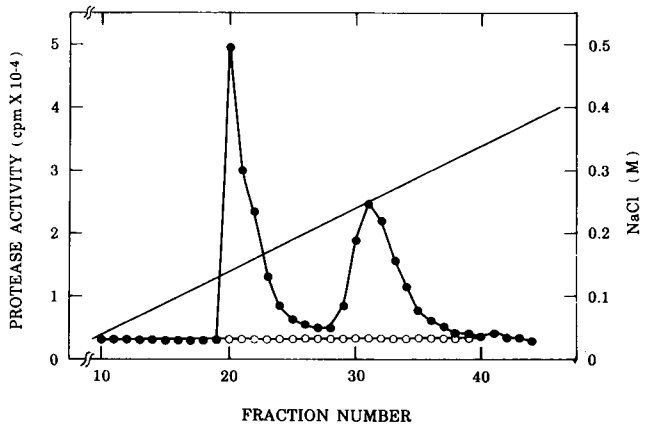

Fig. 2 Isolation of calpain from KM3 cells. Calpain from KM3 Cells was purified by DE-52 column chromatography. Calpain activity was assayed as described under "MATERIALS AND METHODS." (●—●), calpain activity in the presence of 0.2 mM $CaCl_2$; (○—○), calpain activity in the presence of 0.5 mM EGTA; (——), NaCl.

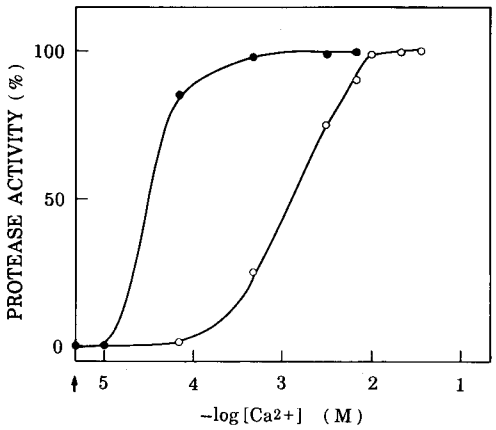

Fig. 3 Calcium requirement of calpain fractions purified from KM3 cells. Calpain fractions purified from a DE-52 column were further purified by Sephacryl S-300 column chromatography and assayed at various concentrations of $CaCl_2$ as described under "MATERIALS AND METHODS." The reaction mixture contained EGTA and EDTA derived from the purified calpain preparations (final concentrations of 0.3 mM each). (●—●), calpain activity of peak I; (○—○), calpain activity of peak II. Arrow indicates 0.5 mM EGTA.

It has been shown that PKM is produced when PKC is incubated with calpain I or calpain II, and is active without addition of Ca^{2+}, phospholipid, and diacylglycerol. Fig. 4 shows the proteolysis of PKC subspecies from KM3 cells by calpain I or calpain II. Type II PKC was more susceptible than Type III PKC to proteolysis by calpain I. Ptoteolysis of PKC by calpain I was markedly enhanced by the addition of phospholipid and diacylglycerol. Both type II and type III PKC were more resistant to proteolysis by calpain II.

Fig. 5 shows the translocation and down-regulation of PKC subspecies in KM3 cells. In the control cells PKC was found in the cytosol but not in the particulate fraction (fig. 5A and 5F). After TPA treatment type II and type III PKC in the cytosol were rapidly decreased. Type II was translocated to the particulate fraction (approximately 15% of the activity in the untreated cytoplasmic fraction) at 2 min (fig. 5B and 5G). At 15 min, type II PKC disappeared in both soluble and particulate fractions, whereas type III PKC translocation to the particulate fraction reached the maximum level (approximately 20% of the type III PKC activity in the untreated cytoplasmic fraction) (fig. 5D and 5I).

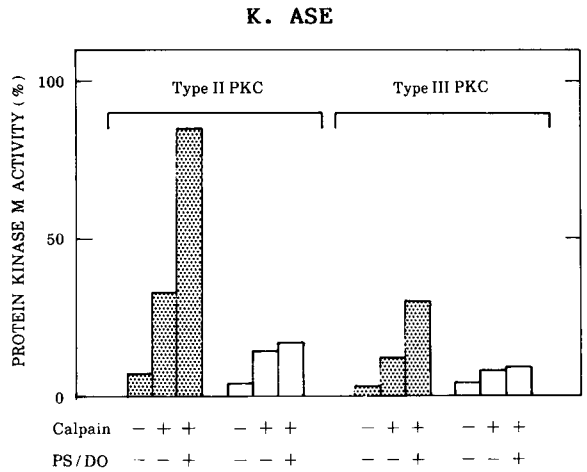

Fig. 4 Proteolysis of PKC by calpain purified from KM3 cells. Either type II or type III PKC was hydrolysed by calpain I(▨) or calpain II(□). The reaction was carried out in the presence or absence of phospholipid, and diolein as described under "MATERIALS AND METHODS."

At 3 hours, a small amount of type III PKC (approximately 1%) remained in the particulate fraction (fig. 5J), and at 6 hours there was no detectable PKC in any fractions. These profiles are more quantitatively plotted in fig. 6.

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

DISCUSSION

Previous studies have shown that treatment with TPA elicits translocation and down-regulation (depletion) of PKC in a variety of cell types.[4,5] 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. Although KM3 cells contain both calpain I and calpain II, this depletion of PKC molecules is presumably initiated by calpain I action, since this protease preferentially cleaves the active form, but not the inactive resting form, of PKC. Also type II PKC is more susceptible than type III PKC to calpain I *in vitro*. Recent analysis

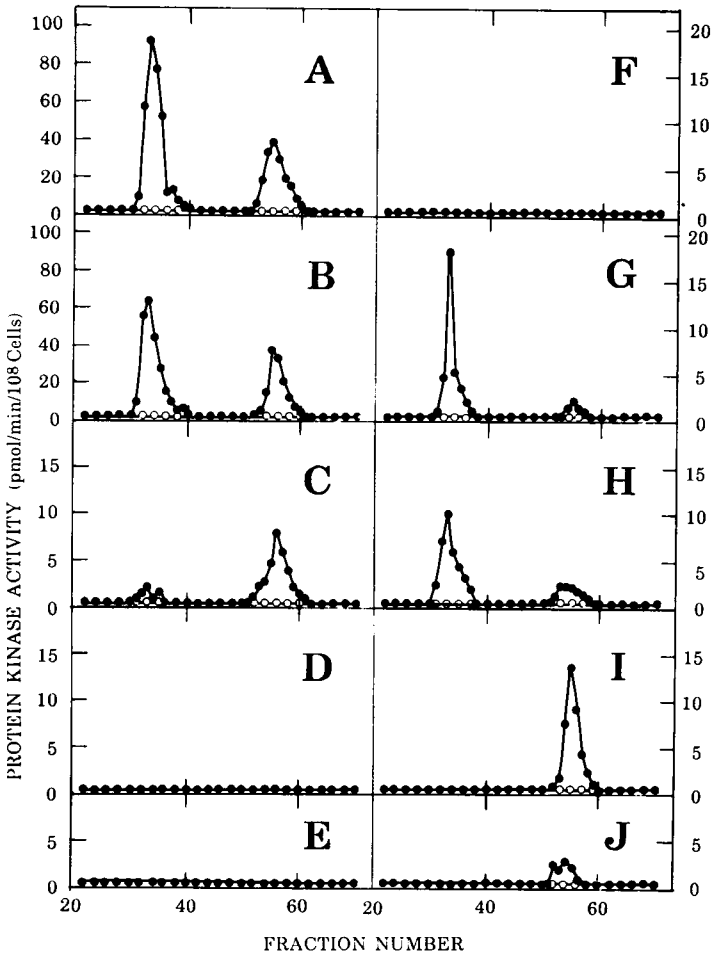

Fig. 5 Translocation and down-regulation of PKC subspecies in KM3 cells. KM3 cells were treated with 50 mM TPA for the indicated times. PKC subspecies were isolated and assayed as described under "MATERIALS AND METHODS." (A-E), activity of PKC subspecies in the cytosol fraction; (F-J), activity of PKC subspecies in the particulate fraction. (A and F), at time 0, (B and G), 2 min; (C and H), 5 min; (D and I), 15 min; (E and J), 180 min after TPA treatment. (●—●), PKC activity in the presence of phosphatidylserine, diolein and $CaCl_2$; (○—○), in the presence of EGTA instead of phosphatidylserine, diolein and $CaCl_2$.

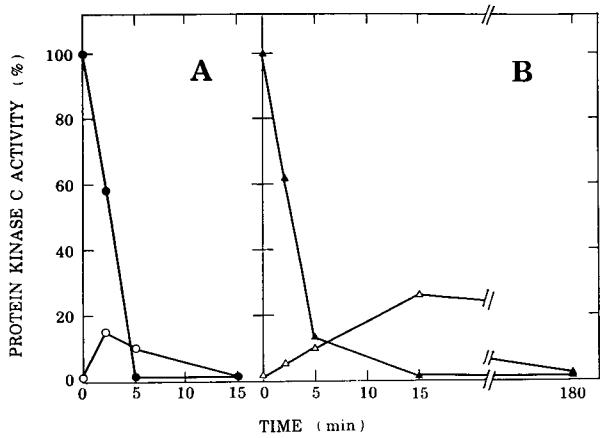

Fig. 6 Time course of translocation and down-regulation of PKC subspecies in KM3 cells. KM3 cells were treated with TPA, and PKC activity in each fraction was analyzed as described in figure 1. (A), type II; (B), type III; (●) and (▲), PKC activity of the cytosol fraction; (○) and (△), PKC activity of the particulate fraction.

indicates that the limited proteolysis of PKC takes place at a site in the third variable region, V3,[8] which connects the regulatory and protein kinase domains of the PKC molecule. There are two alternative possibilities for the significance of this proteolysis. Firstly, that it may be a process to activate PKC and that the resulting fragment may play some roles in the control of cellular functions,[3,12] and secondly, that it is a process to initiate the degradation of PKC, eventually leading to its depletion from the cell, termed down-regulation.[8,21] In support of the latter argument, treatment of KM3 cells with TPA, Ca^{2+}-, phospholipid-, and diacylglycerol-independent protein kinase acitivity 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 C activity originally found in intact KM3 cells is rapidly depleted and disappears after treatment with TPA. Whatever the reason, the limited proteolysis of PKC, generating PKM and/or depletion of PKC, may be an important step of the signal transduction through PKC. It is necessary to identify the true endogeneous substrates of PKC and probably PKM to determine the precise physiological role of this limited proteolysis.

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