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STUDIES ON PROTEOLYSIS OF PROTEIN KINASE C WITH CALPAIN I AND II*

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INDEXING WORDS

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SYNOPSIS

Earlier reports from this laboratory have shown that protein kinase C (PKC) is cleaved with Ca^{2+}-dependent neutral protease (calpain) I or II to produce a catalytically active fragment, and that calpain I, which is active in the micromolar range of Ca^{2+}, may react preferentially with the active form of PKC that is associated with membranes. Subsequently, PKC is shown to exist as a large family of multiple subspecies with subtle individual characteristics. Three types of PKC designated types I, II, and III are purified from rat brain cytosol, which are shown to correspond to the cDNA clones γ, β, and α, respectively. The aim of the present study was to characterize the proteolysis of each PKC

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Abbreviations used are: PKC, proteinkinase C; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis (β-amino ethyl ether)-N,N,N',N'-tetraacetic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

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subspecies with calpain I and II. All types of PKC (82 kDa) were converted to two major fragments: a 47-49-kDa catalytic and a 36-kDa regulatory fragments by the cleavage with either calpain I or II. Analysis of the NH_2-terminal sequence of the resulting catalytic fragments indicated that both calpain I and II cleaved at one or two specific sites in the variable region (V_3) of each PKC molecule of which structure was clearly different among PKC subspecies. From kinetic studies, the cleavage of PKC subspecies with calpain I, and to a lesser extent, with calpain II (active in the millimolar range of Ca^{2+}), was remarkably enhanced by the simultaneous presence of phospholipid and diacylglycerol or phorbol ester, suggesting that the active forms of PKC subspecies were the preferred targets for proteolysis. Whereas, stimulatory abilities of the lipids were variable among PKC subspecies and inactive form of type I PKC was cleaved with calpain I at a significant rate. Quantitative analysis with a fixed amount of calpain under comparable conditions showed that the susceptibilities of the PKC subspecies were distinctly different one another; the relative rates of cleavage of types I, II, and III PKC with calpain I and II were approximately 100:16:2 and 100:48:23, respectively. These results indicated that within the cell various PKC subspecies might be cleaved at different rates under different physiological conditions.

INTRODUCTION

Recent molecular cloning and biochemical analysis has indicated that protein kinase C (PKC) is a family of multiple subspecies of the enzyme.[14] Initially, three cDNA clones, α, β(βI and βII), and γ, are shown to be encoded by distinct chromosomes,[4] whereas two cDNA clones, βI and βII, are derived from alternative splicing from a single RNA transcript.[16] These subspecies have a common structure consisting of four conserved (C_1-C_4) and five variable (V_1-V_5) regions. The third variable region (V_3) connects the regulatory and protein kinase domains.[7] PKC from rat brain tissue is resolved into three subfractions, types I, II, and III, which correspond to γ, β, and α-PKC, on hydoxyapatite column chromatography, respectively.[5,18] Various members of the PKC family show slightly different mode of activation and substrate specificities, and distinctly different tissue

distribution.[14] This multiplicity has been proposed to contribute to diverse roles of PKC in physiological cellular responses to external signals.

It has been reported that PKC is cleaved with Ca^{2+}-dependent neutral protease (calpain) II (active in the millimolar range of Ca^{2+}) to produce catalytic and regulatory fragments.[6] Furthermore it has been documented that calpain I, which is active in the micromolar range of Ca^{2+} concentration, cleaves preferentially the active form of PKC that is associated with membranes, whereas PKC is cleaved with calpain II irrespective of the presence of and absence of phospholipid and diacylglycerol.[9] The present study is designed to characterize and compare proteolysis of PKC subspecies with calpain by examining dependence of calpain I and II on phospholipid, diacylglycerol and phorbol ester, and by determining the site of clevage of PKC subspecies with calpain. Available evidence indicate that the three forms of PKC subspecies are cleaved at one or two specific sites in V_3 region in a different manner.

MATERIALS AND METHODS

Enzyme and assays

PKC was purified from rat brain soluble fraction, and separated into three distinct fractions, types I, II, and III, by hydroxyapatite column chromatography as described.[18] The PKC preparations were practically homogenous on sodium dodecyl sulfate /polyacrylamide gel electrophoresis (SDS/PAGE). PKC was routinely assayed by measuring the incorporation of ^{32}Pi into H1 histone form [γ-^{32}P]ATP.[18] The reaction mixture (0.25 ml) contained Tris/HCl at pH 7.5 (5 μmol), calf thymus H1 histone (50 μg) [γ-^{32}P]ATP (2.5 nmol, 6-12 x 10^4 cpm), magnesium acetate (1.25 μmol), $CaCl_2$ (25 nmol), phosphatidylserine (2 μg), diolein (0.2 μg), and PKC to be assayed. After incubation for 2 min at 30C, the reaction was terminated by the addition of 25% (w/v) trichloroacetic acid, and acid-precipitable materials were collected on Toyo-Roshi membrane filter) pore size, 0.45 μm). When PKC was subjected to the cleavage with calpain I or II, a catalytic fragment was produced that was not affected by Ca^{2+}, phospholipid, and diacylglycerol. Thus, this fragment was assayed with H1

histone as substrate in the presence of EGTA (0.5 mM at final concentration) instead of Ca^{2+}, phospholipid, and diacylglycerol. Homogenous preparations of calpain I and II were prepared from rat kidney by the method of Yoshimura et al.,[22] and assayed routinely with ^{125}I-casein as substrate[9] except that Triton X-100 (0.015% (v/v) at final concentration) and Tween 20 (1% (v/v) at final concentration) were added to the reaction mixture.

Other materials and chemicals

Calf thymus H1 histone was prepared by the method of Oliver et al.,[15] Erythrocyte membrane phopholipid was extracted and fractionated as described.[8] Phosphatidylserine (bovine brain) and diolein were purchased from Serdary Research Laboratories. Bovine serum albumin (fatty acid-free), casein (Hammarsten), 12-*O*-tetradecanoylphorbol-13-acetate (TPA), [γ-^{32}P]ATP, and Superose 12 HR 10/30 column were obtained from Armour, E. Merck, Chemicals for Cancer Research, Du Pont-New England Nuclear, and Pharmacia LKB Biotechnology, respectively.

Proteolysis of PKC with calpain I and II

The reaction mixture (0.1 ml) initially contained Tris/HCl at pH 7.5 (2.5 μmol), 2-mercaptoethanol (0.5 μmol), Triton X-100 (0.015% (v/v) at final concentration), Tween 20 (1% (v/v) at final concentration), PKC (2.5 μg), and calpain I or II to be be assayed. Where indicated, fatty acid-free bovine serum albumin (200 μg) was added. Although Triton X-100, Tween 20, and albumin did not interfere with proteolysis at these concentrations, they apparently stabilized PKC during incubation. Phospholipid, diolein, and TPA were added as indicated in each experiment. The reaction was started by the addition of $CaCl_2$ (0.2 mM at final concentration for calpain I, and 0.5 mM at final concentration for calpain II). After incubation at 20C for various times as indicated, the reaction was stopped by the addition of 10 μl of 25 mM EGTA. Alternatively, when indicated, a 10 μl aliquot of the reaction mixture was taken and immediately assayed for the active fragment of PKC as specified above.

Amino acid sequence analysis

Samples (3-5 μg of protein for each lane (5 mm width)) were

loaded onto minigels (9 x 9 cm, 1 mm thick) containing a 10% polyacrylamide gel, and electrophoresed as described by Laemmli.[11] After electrophoresis, the protein in the gel was transferred to a polyvinylidene difluoride membrane (Millipore), and then directly employed for amino acid sequence analysis by the method of Matsudaira[12] with an Applied Biosystems Model 477A protein sequenator, equipped with an on-line Model 120A analyzer, using the manufacture's standard program.

Other procedures

The monoclonal antibody designated CKmC1β-a was raised against the synthetic polypeptide, which corresponds to the amino acid sequence from residues 20 to 39 (FARKGALRQKNVHEVKNHKF) deduced from the β-cDNA clone, as described previously.[10] Lipids were dispersed in 20 mM Tris/HCl at pH 7.5 by sonication as described previously.[8] The radioactive phosphate was determined by counting of Cerenckov radiation. Protein was determined by SDS/PAGE, Coomassie Brilliant Blue staining, followed by densitometric tracing, with bovine serum albumin as a standard by the method of Weber et al.[21]

RESULTS

Identification of the catalytic and regulatory fragments

Types I, II, and III PKC all produced a catalytically fully active fragment which was previously referred to as protein kinase M, when incubated with either calpain I or II. This active fragment can be distinguished from the native PKC by assaying with H1 histone as substrate in the presence of EGTA instead of Ca^{2+}, phospholipid, and diacylglycerol. Figure 1 shows a typical example where type II PKC was cleaved with calpain II followed by the separation of the resulting fragments on gel filtration. Both the enzymatic activity of the catalytic fragment and proteins of each column fraction were determined. The intact PKC (82 kDa) disappeared, and Ca^{2+}-, phospholipid-, and diacylglycrol-independent enzyme (catalytic fragment, 46 kDa) and an additional protein fragment (regulatory fragment, 36 kDa) were produced. This regulatory fragment was recognized as intact PKC by the monoclonal antibody, CKmC1β-a, as judged by immunoblot analysis after SDS

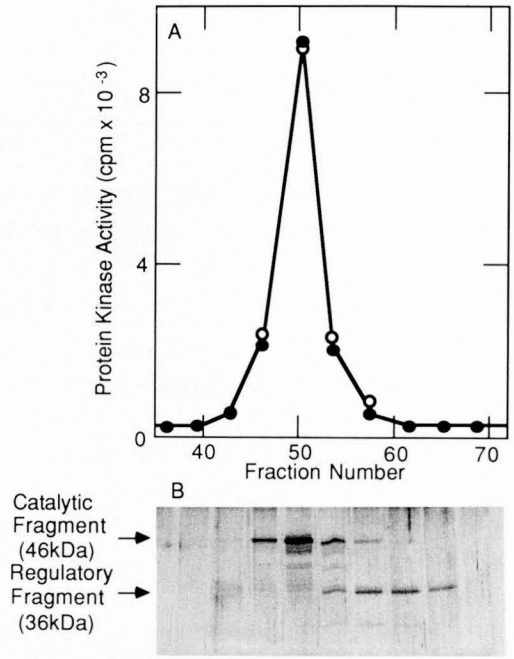

Fig. 1 Analysis of fragments of PKC generated by calpain.

Type II PKC (12 μg) was incubated for 30 min at 20C with calpain II (4 μg) in the mixture (0.4 ml) containing Tris/HCl at pH 7.5 (10 μmol), $CaCl_2$ (0.2 μmol), 2-mercaptoethanol (2 μmol), phosphatidylserine (4 μg) plus diolein (0,4 μg), Triton X-100 (0.015% (v/v) at final concentration), and Tween 20 (1% (v/v) at final concentration). Albumin was not added to the reaction mixture. Then, the reaction was terminated by the addition of EGTA (2.6 mM at final concentration). The reaction mixture was loaded directly onto a Superose 12 HR 10/30 column, which was connected to a Fast Protein Liquid Chromatography system (Pharmacia) equilibrated beforehand with 20 mM Tris/HCl at pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 10 mM 2-mercapto-ethanol, 0.02% Triton X-100, and 0.5 M NaCl at 4C. Elution of proteins was performed with the same solution at a flow rate of 0.3 ml/min, and fractions of 0.3 ml each were collected. A, protein kinase activity. A 10-μl aliquot of each fraction was assayed for protein kinase with H1 histone as substrate in the presence (●) or absence (○) of Ca^{2+}, phosphatidylserine, and diolein. B, protein analysis. A 20-μl aliquot of each fraction was employed for SDS/10% PAGE using conditions described under "MATERIALS AND METHODS" followed by silver staining of protein. The gel was calibrated with the following standards: myosin, 205 kDa; β-galactosidase, 116 kDa; phosphorylase *b*, 98 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; and carbonic anhydrase, 29 kDa.

/PAGE (data not shown). With various combinations of PKC subspecies and calpain I and II, essentially similar results were obtained as summarized in Tables 1 and 2. Some combinations produced two bands of catalytic fragments of the PKC molecule while all combinations generated a single homogenous 36 kDa of regulatory fragment.

Determination of sites of cleavage of PKC molecules with calpain

Tables 1 and 2 summarize estimation of the molecular mass of fragments produced in various combinations of proteolysis of PKC subspecies with calpain I and II, respectively. The amino acid sequences of NH_2-terminal of catalytic fragments of PKC subspecies generated by either calpain I or II were determined using a high sensitibity gas-phase sequenator. For all samples, 2-6 pmol of amino acid derivative was repeatedly obtained, and the sequence of more than 10 amino acid residues was established which coincided exactly with the part of the predicted sequence located at the V_3 region. Figure 2 summarizes the sites determined in various combinations of PKC subspecies and calpain I or II. In all cases,

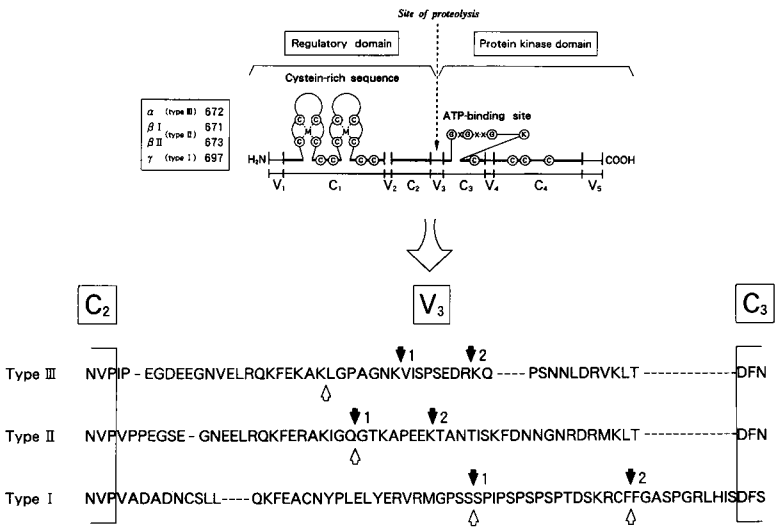

Fig. 2 Common structure of α-, βI-, βII-, and γ-PKC subspecies and sites of cleavage of PKC molecules with calpain I and II.

C_1-C_4 and V_1-V_5 indicate conserved and variable regions, respectively. The numbers in the square show the number of amino acid residue composing each subspecies. C, cysteine; G, glycine; K, lysine; M, metal; and X, any amino acid. Hyphens are introduced for optimal alignment. Arrows indicate sites of cleavage. (⇩), with calpain I; (⇧), with calpain II. Numbers 1 and 2 attached to arrows represent Site 1 and Site 2, respectively.

Table 1 Automated Edman degradation of the catalytic fragments of PKC produced by calpain I.

Each of types I, II, and III PKC (12.5 μg) was incubated with calpain I (1.5 μg for types I and II PKC, and 12 μg for type III PKC) under similar conditions to those described in the legend to Fig. 1. After the reaction was completed, the proteins were precipitated in methanol, isolated, and sequenced as described under "MATERIALS AND METHODS". Although more than 10 amino acid residues were established for each fragment, 6 amino acid residues are given in this table. The numbers in parentheses indicate the yields of phenylthiohydantoin derivatives at each cycle. The sequences are indicated using one letter abbreviations. The amino acids in parentheses are deduced from the cDNA sequences; the phenylthiohydantoin derivatives could not be estimated by sequence analysis.

	N-terminal amino acid sequence of catalytic fragment (pmol)					
Cycle Number	49 kDa fragment (Type I)	47 kDa fragment (Type I)	46kDa fragment (Type II)	45kDa fragment (Type II)	49kDa fragment (Type III)	47kDa fragment (Type III)
1	S (1.6)	F(2.5)	Q(3.0)	T(8.1)	V(2.2)	K(5.0)
2	P(2.9)	G(1.4)	G(5.7)	A(6.2)	I(3.4)	Q(4.6)
3	I(2.7)	A(1.7)	T(4.1)	N(4.7)	S(1.7)	P(4.8)
4	P(1.7)	(S)	K(2.1)	T(6.2)	P(3.3)	S(2.9)
5	S(1.1)	P(2.1)	A(3.6)	I(4.8)	(S)	(N)
6	P(1.6)	G(1.1)	P(1.6)	S(3.2)	E(2.0)	N(3.0)

Table 2 Automated Edman degradation of the catalytic fragments of PKC produced by calpain II.

Each of types I, II, and III PKC (12.5 μg) was incubated with calpain II (1.5 μg). Detail experimental conditions and procedures are given in Table 1.

	N-terminal amino acid sequence of catalytic fragment (pmol)			
Cycle Number	49kDa fragment (Type I)	47kDa fragment (Type I)	46kDa fragment (Type II)	47kDa fragment (Type III)
1	S (4.5)	F (6.1)	Q (7.0)	L (3.0)
2	P (3.5)	G (7.0)	G (7.3)	G (3.5)
3	I (4.0)	A (3.9)	T (7.0)	P (3.0)
4	P (2.8)	S (7.0)	K (2.7)	A (2.2)
5	S (2.0)	P (2.1)	A (7.0)	G (3.0)
6	P (3.1)	(G)	P (3.8)	N (3.0)

the cleaved sites were located at the V_3 region, which connects the regulatory and catalytic domains. For type III PKC, calpain I and II cleaved it at different sites. There was no obvious common sequence around the cleavage sites of various PKC subspecies. This may be a major reason for the different phospholipid requirements and susceptibilities of PKC subspecies to proteolysis with calpain (see below). A single homogenous regulatory fragment always appeared whereas two catalytic fragments were often obtained. Appearance of the larger catalytic fragment was faster than that of smaller one, and the amount of the larger one was decreased after prolonged proteolysis. This suggests that of the two sites cleaved, Site 1 was attacked first, and then the smaller catalytic fragment was generated by cleavage at Site 2 (Fig. 2).

Comparison of effect of phospholipid, diacylglycerol, and TPA on proteolysis of PKC

The next set of experiments were conducted to investigate the effect of phospholipid, diacylglycerol and TPA on proteolysis of PKC subspecies with calpain I and II. Time course of the formation of a fully catalytically active fragment from PKC subspecies by proteolysis with calpain is shown in Fig. 3. Confirming the previous observations with unfractionated PKC, the reaction catalyzed by calpain I was markedly enhanced by the simultaneous presence of phospholipid and dacylglycerol. Type I PKC was cleaved to some extent with calpain I in the absence of phospholipid and diacylglycerol differently from types II and III (Fig. 3A). Either diolein or phospholipid alone showed practically no effect. On the other hand, the proteolysis of PKC subspecies with calpain II was not remarkably affected by the simultaneous presence of phospholipid and diolein (Fig. 3D). Essentially similar results were obtained for proteolysis of types II and III PKC with calpain II (data not shown). TPA could substitute for diacylglycerol in any combination of proteolysis. A series of analyses indicated that both phospholipid and diacylglycerol acted on the substrate protein that was PKC rather than the protease itself. When casein was used as a substrate, neither calpain I nor II required phospholipid and diacylglycerol for maximum enzymatic activity (data not shown), indicating that calpain, in particular calpain I, preferentially cleaved the active form of PKC, whose conformation probably differed from that of the inactive form.

Relative susceptibility of PKC subspecies to calpain

Quantitative analysis with a fixed amount of calpain under the comparable condition given in Fig. 4 indicates that type I PKC was most susceptible of all types of PKC to proteolysis with both calpain I and II whereas type III PKC was far more resistnat to the proteolysis. The relative rates of cleavage of types I, II, and III PKC with calpain I were approximately 100:16:2, respectively, while those with calpain II were approximately 100:48:23, respectively. These results indicate that there is a difference in susceptibility to calpain among the three subspecies of PKC.

DISCUSSION

In the present study, we have clarified the characteristics of proteolysis of PKC subspecies with calpain I and II. There are differences in dependence on phospholipid and diacylglycerol, in susceptibility to proteolysis, and in sites of cleavage among various combinations of PKC subspecies and calpain. It is worthy to note that this proteolytic activation process, particularly by calpain I, is markedly enhanced by the simultaneous addition of Ca^{2+}, phospholipid, and diacylglycerol or phorbol ester, which normally activate PKC. It has been postulated that diacylglycerol or phorbol ester remains to be associated with the cellular membrane and recruits PKC to the membrane, where a quaternary complex consisting of PKC, Ca^{2+}, phospholipid, and diacylglycerol or phorbol ester is composed. It is suggested that the conformation of the PKC in the quaternary complex differs from that of inactive free form and is the active form is preferentially cleaved with calpain, particularly with calpain I. However, type I PKC can be cleaved to some extent with calpain I in the absence of phospholipid and diolein. Type I PKC, which appears to be expressed only in central nervous tissues and to exist in both cell membrane and cytoplasm of neurons such as pyramidal cell and purkinje cell,[14] may be cleaved with calpain I even in cytoplasm where it exists as an inactive form (not membrane-bound form).

Although the physiological significance of the proteolysis is still unclear, there are two possibilities of the role of proteolysis of PKC with calpain. First, this proteolysis may be a process to activate PKC, and the resulting active fragment may be

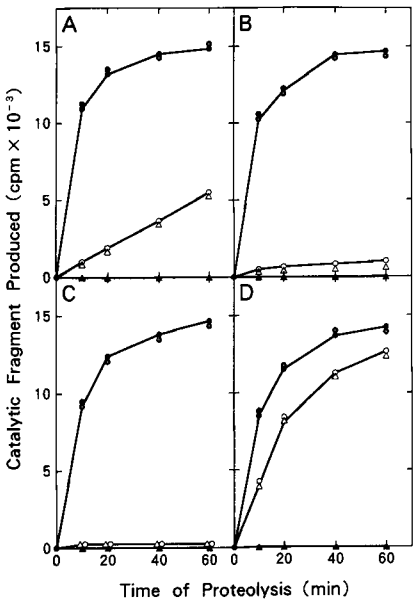

Fig. 3 Effect of phospholipid, diacylglycerol, and TPA on proteolysis of PKC subspecies with calpain I and II.

A, B, and C, proteolysis of types I, II and III PKC with calpain I, respectively. Each of PKC subspecies (2.5 μg) was preincubated in the mixture (0.1 ml) with calpain I at 20C in the presence or absence of phospholipid (10 μg) plus diolein (0.1 μg) or TPA (200 nM at final concentration) under the standard condition, except that fatty acid-free bovine serum albumin (200 μg) was added to the reaction mixture. Calpain I, 0.05 μg, 0.3 μg, and 2.5 μg were employed for types I, II, and III PKC, respectively. At the various times indicated, a 10-μl aliquot of the mixture was taken and immedeately assayed for the active enzyme fragment produced from PKC in the presence of EGTA. In control experiments calpain was omitted. D, proteolysis of type II PKC with calpain II. Type II PKC subspecies (2.5 μg) was preincubated in the mixture (0.1 ml) with calpain II (0.3 μg) at 20C in the presence or absence of phospholipid (10 μg) plus diolein (0.1 μg) or TPA (200 nM at final concentration). Catalytic fragment produced in the presence of phospholipid plus diolein (●), phospholipid plus TPA (◎), or phospholipid alone (○), in the absence of neither of phospholipid, diolein nor TPA (△), in the absence of calpain (▲).

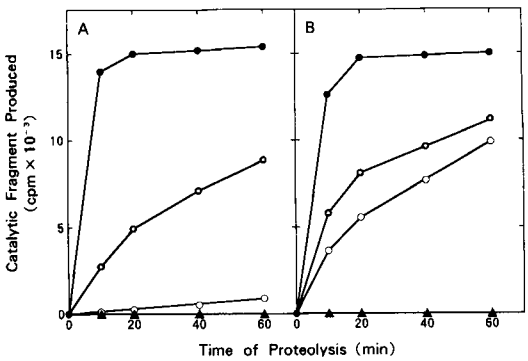

Fig. 4 Relative susceptibility of types I, II, and III PKC to calpain I and II.

Types I, II, and III PKC (2.5 μg each) are separately subjected to proteolysis with a fixed amount of calpain I or II (0.8 μg) at 0C in the presence of Ca^{2+}, phospholipid, and diolein as described in the legend to Fig. 3. At the various times indicated, an aliquot of the mixture was taken and immediately assayed for the catalytically active fragments produced in the presence of EGTA. In control experiments calpain I or II was omitted. A, with calpain I; B, with calpin II.
●, cleavage of type I; ◎, cleavage of type II; ○, cleavage of type III; ▲, minus calpain.

released from membranes and play some roles in the control of cellular function.[13,20] Second, in contrast, this may be a process to initiate the degradation of PKC, eventually depleting the enzyme from the cell. It has been reported that in a variety of tissues and cell types, TPA, the phorbol ester which induces persistent activation of PKC, elicits its translocation from the cytosol to the membrane, and subsequent depletion, designated down-regulation of PKC.[2,3,17,19,23] Calpain seems to initiate the down-regulation of PKC since PKC forms a quaternary complex with TPA when translocated from cytosol to the membrane and becomes a preferred target for calpain. Upon treatment of TPA, various subspecies of PKC co-expressed in a single cell type, such as KM3, disappear at different rates,[1] which concide with the rates of the *in vitro* proteolysis. This difference in susceptibility to proteolysis may be caused by the difference in sites of cleavage with calpain amount PKC subspecies. It is most likely that the limited proteolysis of the PKC molecules with calpain I is directly related to the initiation of degradation of the enzyme, and that various subspecies of PKC are depleted from the cell at different rates. On the other hand, the involvement of calpain II in down-regulation of PKC has not yet been substantiated, since this protease requires the millimolar range of Ca^{2+} for the activity, and there is no evidence thus far that the protease cleaves selectively the active form of PKC.

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