Limited proteolysis of three distinct subspecies of protein kinase C (Ca\(^2+\)/phospholipid-dependent enzyme, PKC), types I (γ), II (βI and βII), and III (α), with Ca\(^{2+}\)-dependent neutral proteases I and II (calpains I and II) was studied. All forms of PKC (82 kDa) were converted to two major fragments: a 45–49-kDa catalytic fragment and a 36-kDa regulatory fragment. The cleavage of these PKC subspecies by calpain I (active in the micromolar range Ca\(^{2+}\)/ion concentration), and, to a lesser extent, by calpain II (active in the millimolar range), was enhanced by the simultaneous presence of phospholipid and diacylglycerol or phorbol ester, suggesting that the activated form of PKC is the preferred target for proteolysis. Analysis of the NH\(_2\)-terminal sequence of the resulting catalytic fragments indicated that both calpains I and II cleave at one or two specific sites in the third variable region (V\(_3\)) of each PKC molecule. Under comparable conditions with calpains I and II, the relative rates of cleavage of types I, II, and III PKC were approximately 100:16:2 and 100:48:23, respectively. The results imply that within the cell various PKC subspecies may be cleaved at different rates under different physiological conditions.

Recent molecular cloning analysis indicates that protein kinase C (PKC) exists as a family of multiple subspecies; four rat brain cDNA clones named α-, βI-, βII-, and γ-PKC were initially identified (for a review, see Ref. 1). 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\(_3\)) connecting the regulatory and protein kinase domains (2). Recently, another group of cDNA clones designated δ-, ε-, and ζ-PKC have been isolated from a rat brain cDNA library (3). Rat brain PKC has been resolved into three fractions, types I, II, and III, which correspond to γ-, β- (βI and βII), and α-PKC, on hydroxyapatite column chromatography (4, 5). Although δ-, ε-, and ζ-PKC have not yet been identified, various members of the PKC family show subtly different enzymatic properties and distinctly different tissue distribution (1). Earlier reports from this laboratory (6, 7) have shown that PKC is cleaved by a Ca\(^{2+}\)-dependent neutral protease (calpain) to produce a catalytically active fragment (protein kinase M), and that calpain I, which is active in the micromolar range of Ca\(^{2+}\) concentration, may react preferentially with the active form PKC that is associated with membranes. The present studies were undertaken to identify the sites of cleavage of PKC by calpain. The results presented indicate that the three forms of the enzyme, α-, β-, and γ-PKC, are cleaved at one or two specific sites in the V\(_3\) region at different rates.

**EXPERIMENTAL PROCEDURES**

**Enzymes and Assays**—PKC was purified from rat brain and separated into three distinct fractions, types I, II, and III, by hydroxyapatite chromatography (5). The PKC preparations were practically pure on SDS/PAGE. PKC was routinely assayed with H1 histone as substrate in the presence of 0.1 mM Ca\(^{2+}\), 8 μg/ml phosphatidylserine, and 0.8 μg/ml diolein (5). The reaction mixture (0.25 ml) contained Tris/HCl (5 μmol), pH 7.5, calf thymus H1 histone (50 μg), [γ-\(^{32}\)P]ATP (2.5 nmol, 6–12 \(\times\) 10\(^5\) cpm), magnesium acetate (1.25 μmol), CaCl\(_2\) (25 μmol), phosphatidylserine (2 μg), diolein (0.2 μg), and the enzyme to be assayed. After incubation for 2 min at 30 °C, the reaction was terminated by the addition of 25% trichloroacetic acid. Then, acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm). When the enzyme was digested with calpain, a catalytic fragment was produced that was not affected by Ca\(^{2+}\), phospholipid, and diacylglycerol. Thus, this fragment was selectively assayed with H1 histone as substrate in the presence of EGTA (0.5 mM final concentration) instead of Ca\(^{2+}\), phospholipid, and diacylglycerol. Homogenous preparations of calpain I (active in the micromolar Ca\(^{2+}\) range), and calpain II (active in millimolar Ca\(^{2+}\) range), were obtained from rat kidney by the method of Yoshimura et al. (8).

**Other Materials and Chemicals**—Calf thymus H1 histone was prepared by the method of Oliver et al. (9). Erythrocyte membrane phospholipids were extracted and fractionated as described (7). Phosphatidylserine (bovine brain) and diolein were purchased from Serdary Research Laboratories. Bovine serum albumin (fatty acid-free), [γ-\(^{32}\)P]ATP, and Superose 12 HR 10/30 column were obtained from Amour, Du Pont-New England Nuclear, and Pharmacia LKB Biotechnology Inc., respectively.

**Proteolysis of PKC with Calpain**—The reaction mixture (0.1 ml) initially contained Tris/HCl (2.5 μmol), pH 7.5, 2-mercaptoethanol (0.5 μmol), Triton X-100 (0.015% at final concentration), Tween 20 (1% at final concentration), PKC (2.5 μg), and calpain. Where indicated, fatty acid-free bovine serum albumin (200 μg) was added. Triton X-100, Tween 20, and albumin at these concentrations did not interfere with proteolysis, but apparently stabilized PKC during incubation. Phospholipid and diolein were added as indicated in each experiment. The reaction was started by the addition of CaCl\(_2\), at 0.2 mM final concentration for calpain I or at 0.5 mM final concentration for calpain II. After incubation at 20 °C 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 × 9 cm, 1 mm thick) containing a 10% polyacrylamide gel, and electrophoresed as described by Laemmli (10). After electrophoresis, the protein in the gels was transferred to a polyvinylidene difluoride membrane (Millipore), and then directly employed for amino acid sequence analysis by the method of Matsudaira (11) with an Applied Biosystems Model 477A protein sequenator, equipped with on-line Model 120A analyzer, using the manufacturer’s standard program.

**Other Procedures**—Lipids were dispersed in 20 mM Tris/HCl, pH 7.5, by sonication and employed as described previously (7). 32P was determined by scintillation counting of Cerenkov radiation. Concentration of enzymes was determined by SDS/PAGE, Coomassie Brilliant Blue staining, followed by densitometric tracing, with bovine serum albumin as standard by the method of Weber et al. (12).

**RESULTS**

Consistent with previous observations with a mixture of PKC subtypes (6, 7), types I, II, and III PKC each produced a fragment which was fully active without added Ca2+, phospholipid, and diacylglycerol, when incubated with either calpain I or II. The experiment given in Fig. 1 shows the time course of the formation of a fully catalytically active fragment from type II PKC. It is worth noting, as described earlier (7), that the reaction catalyzed by calpain I is markedly enhanced by the simultaneous presence of phospholipid and diacylglycerol (Fig. 1A). Dipeol alone showed practically no effect (data not shown). The conversion of PKC to the catalytic fragments was stimulated by the addition of diol to the phospholipid, which alone was much less effective, as described previously (7). This characteristic feature was less evident when calpain II was used (Fig. 1B). Diamylglycerol could be replaced by TPA (data not shown). Using cascin as substrate, neither calpain I nor II required phospholipid and diacylglycerol for maximum enzymatic activity (data not shown), indicating that calpain, particularly calpain I, preferentially cleaves the active form of PKC, whose conformation probably differs from that of the inactive form. Essentially similar results were obtained for types I and III PKC, although the reaction rate was different (see below).

The next set of experiments was conducted to identify the catalytic and regulatory fragments after digestion with calpain, and one typical example is shown in Fig. 2. Here, type II PKC was incubated with calpain II as described in the legend, and the reaction mixture was subjected to gel filtration. Each column fraction was assayed for protein by SDS/PAGE analysis (Fig. 2A) and for enzyme activity (Fig. 2B). The intact PKC (82 kDa) disappeared, and a Ca2++-, phospholipid-, and diacylglycerol-independent enzyme fragment (46 kDa) and an additional protein fragment (36 kDa) were produced. The latter was recognized as intact PKC by an antibody raised against the NH2-terminal C1 region of type II PKC (FARKGALRQKVNHEVKKHK+4) as judged by immunoblot analysis after SDS/PAGE (data not shown). The results of various combinations of PKC subtypes and calpain I or II are summarized in Fig. 3 and Table I. Some

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![Fig. 1. Proteolysis of type II PKC by calpains I and II.](image)

![Fig. 2. Analysis of fragments of PKC generated by calpain.](image)
Fig. 3. Proteolysis of PKC subspecies by calpains I and II. A, PKC subspecies (2.5 µg each) was incubated for 30 min in the mixture (0.1 ml) containing either calpain I (0.3 µg) or II (0.3 µg), phosphatidylserine (1 µg), and diolein (0.1 µg) under conditions similar to those given in Fig. 1, except that for type III PKC calpain I (2.5 µg) or II (0.3 µg) was used. Albumin was omitted from the reaction mixture. Then, a 20-µl aliquot of each reaction mixture was taken and subjected to electrophoresis by SDS, 10% PAGE, followed by protein staining with Coomassie Brilliant Blue. The molecular mass of each fragment was estimated by calibrating the gel as described in the legend to Fig. 1, except that for type III PKC calpain I (2.5 µg) or II (0.3 µg) was used. Albumin was omitted from the reaction mixture. Then, a 20-µl aliquot of each reaction mixture was taken and subjected to electrophoresis by SDS, 10% PAGE, followed by protein staining with Coomassie Brilliant Blue. The molecular mass of each fragment was estimated by calibrating the gel as described in the legend to Fig. 2. Lanes 1, 2, and 3, fragments generated by calpain I from types I, II, and III PKC, respectively; lanes 4, 5, and 6, fragments generated by calpain II from types I, II, and III PKC, respectively. B, PKC type I (2.5 µg) was incubated as described above except that various amounts of calpain I or II were used. Then, the fragments generated were analyzed as described above. Lanes 1, 2, 3, and 4, with 0.3, 0.15, and 0.075 µg of calpain II, and without calpain II, respectively.

Table I

<table>
<thead>
<tr>
<th>PKC</th>
<th>Calpain</th>
<th>Molecular mass</th>
<th>Catalytic fragment</th>
<th>Regulatory fragment</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>kDa</td>
<td>kDa</td>
</tr>
<tr>
<td>Type I</td>
<td>I</td>
<td>49, 47</td>
<td>36</td>
<td></td>
</tr>
<tr>
<td>Type I</td>
<td>II</td>
<td>49, 47</td>
<td>36</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>Type III</td>
<td>II</td>
<td>47</td>
<td>36</td>
<td></td>
</tr>
</tbody>
</table>

combinations of PKC subspecies and calpain produced two bands with catalytic activity upon SDS/PAGE analysis, due to proteolysis at two sites (see below). This was confirmed by amino acid sequence analysis of the NH2-terminal region of each fragment.

Fig. 4. Automated Edman degradation of the catalytic fragments of PKC. Each type of PKC was incubated with either calpain I or calpain II under the conditions described in the legend to Fig. 3, except that the reaction mixture was scaled up five times. After the reaction was completed, the proteins were precipitated in methanol, isolated, and sequenced under conditions described under "Experimental Procedures." Although more than 10 amino acid residues were established for each fragment, 6 amino acid residues are given here. A and B, 49- and 47-kDa fragments of type I PKC, respectively, generated by calpain I; C and D, 49- and 47-kDa fragments of type I PKC, respectively, generated by calpain II; E and F, 46- and 46-kDa fragments of type II PKC, respectively, generated by calpain I; G, 46-kDa fragment of type II PKC generated by calpain II; H and I, 46- and 46-kDa fragments of type III PKC, respectively, generated by calpain I; J, 46-kDa fragment of type III PKC generated by calpain II. 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. •••••, yields of phenylthiohydantoin derivatives at the first and fourth cycles; O—O, those at the second and fifth cycles; and O—O, those at the third and sixth cycles.
Proteolysis of Protein Kinase C

![Diagram of proteolysis of PKC](image)

**TABLE II**
Relative susceptibility of types I, II, and III PKC to calpains I and II

Types I, II, and III PKC (2.5 μg each) were separately subjected to proteolysis with a fixed amount of calpain I or II (0.8 μg) in the presence of Ca²⁺, phospholipid, and diolein under the conditions given in Fig. 1, except that incubation was performed on ice for 10 min. At low temperatures, the proteolytic reaction proceeded linearly with time for at least 10 min. The catalytically active fragments produced were assayed after the reaction was stopped by the addition of EGTA as described under “Experimental Procedures.” The numbers in parentheses represent relative rates of the proteolysis, taking the rate of proteolysis of type I PKC as 100.

<table>
<thead>
<tr>
<th>PKC</th>
<th>Catalytic fragment produced*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>With calpain I</td>
</tr>
<tr>
<td>Type I</td>
<td>3.1 (100)</td>
</tr>
<tr>
<td>Type II</td>
<td>0.51 (16)</td>
</tr>
<tr>
<td>Type III</td>
<td>0.07 (2)</td>
</tr>
</tbody>
</table>

*One unit of catalytic fragment was defined as that amount of enzyme which incorporated 1 nmol of phosphate from ATP into H₁ histone per min under the standard condition.

That of the 47-kDa fragment with a small amount of calpain II, and the formation of the 49-kDa fragment decreases with increasing amounts of the calpain (Fig. 3B).

Finally, experiments were conducted to examine the relative susceptibility of types I, II, and III PKC to proteolysis. Results summarized in Table II indicate that type I PKC is more susceptible than type II PKC to proteolysis by calpain I, whereas type III PKC is far more resistant and only 2% of type III PKC is cleaved under the same conditions. With calpain II the relative rates of cleavage of types I, II, and III PKC were approximately 100:48:23, respectively.

**DISCUSSION**

It has been repeatedly shown that limited proteolysis of purified PKC yields two major fragments, the regulatory and protein kinase domains, and that this proteolysis may occur in a proposed hinge region, V₃ (7, 14). The present studies unequivocally identified the sites of proteolysis of three forms of PKC, type I (γ), II (β₁ and β₁I), and type III (α) by calpains I and II. All sites of proteolysis are located in the variable region, V₃, which connects the regulatory domain (NH₂-terminal half) and the protein kinase domain (COOH-terminal half). There is no obvious common sequence around the cleavage sites. Quantitative analysis indicates that susceptibilities of the PKC subspecies to proteolysis clearly differ from one another; type I PKC is most rapidly cleaved by both calpains I and II, whereas type III PKC is relatively resistant to proteolysis. It is important to note that calpain I appears to cleave preferentially the active form of the enzyme, because the proteolysis is greatly enhanced by the simultaneous presence of Ca²⁺, phospholipid, and diacylglycerol or phorbol ester, which normally activate PKC.

Although limited proteolysis of PKC may generate a catalytically active fragment, previously called protein kinase M (6, 7), the physiological significance of this proteolysis is not yet fully appreciated. Two alternative possibilities may be considered. First, this proteolysis may activate PKC, and the resulting active fragment may play some role in the control of cellular function (15, 16). Second, by contrast, it may be a process initiating the degradation of PKC, eventually depleting the enzyme from the cell. Recent studies in several laboratories (17–21) have shown that, in a variety of tissues and cell types, TPA, which induces persistent activation of PKC, elicits its translocation from the cytosol to the membrane, and subsequent depletion, termed down-regulation of PKC. However, the catalytically active fragment of the enzyme, protein kinase M, is not always recovered from the cytosol. Recent analysis in this laboratory (22) indicates that, upon treatment with TPA, various subspecies of PKC co-expressed in a single cell type disappear quickly at different rates, which coincide with the rates of the in vitro proteolysis described in this paper. It is attractive to surmise that the limited proteolysis of the PKC molecule by calpain, particularly by calpain I, is directly related to the initiation of down-regulation of the enzyme, and that various types of PKC are depleted from the cell at different rates due to the proteolysis at their variable region, V₃.

**Acknowledgments**—We thank S. Yamaguchi for running the protein sequenator. The skillful secretarial assistance of S. Nishiyama and Y. Gotoh is appreciated.

**REFERENCES**

Proteolysis of Protein Kinase C