Isolation and characterization of δ-subspecies of protein kinase C from rat brain

(protein phosphatase 2A/diacylglycerol/phorbol ester)

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ABSTRACT The δ-subspecies of protein kinase C (δPKC) was purified to near homogeneity from the Triton X-100 extract of the rat brain particulate fraction by successive chromatographies on S-Sepharose fast flow, phenyl 5PW, heparin 5PW, hydroxyapatite, and Mono Q columns. The purified enzyme was a doublet with molecular masses of 78 and 76 kDa on SDS/PAGE. The doublet proteins were separated partially by Mono Q column chromatography; both were recognized by the antibodies raised against synthetic oligopeptides, parts of the deduced amino acid sequence of the rat δPKC. Protein phosphatase 2A treatment suggested that the 78-kDa protein was a phosphorylated form of the 76-kDa protein. To confirm the structural and genetic identity of the doublet proteins, δPKC was expressed in COS 7 cells by transfecting its cDNA-constructed plasmid and was purified for comparison. This recombinant enzyme was also a doublet. The enzymes isolated from the brain and COS 7 cells showed identical reactivities with δPKC-specific antibodies, chromatographic behaviors, and V8 protease peptide mappings. In addition, these two enzyme preparations were indistinguishable from each other in their responses to phosphatidylserine, diacylglycerol, phorbol esters, free fatty acids, Ca2+, and enzyme inhibitors. Comparison was also made between the enzymologic properties of δPKC and αPKC, which were distinctly different from each other.

MATERIALS AND METHODS

Materials and Chemicals. Sprague–Dawley rats (6 weeks old) were used for the present studies. The catalytic subunit of protein phosphatase 2A (PP2A), which was purified from human erythrocytes, was donated by H. Usui and M. Takeda (University of Hiroshima). H1 histone was purified from calf thymus. Myristoylated alanine-rich C-kinase substrate (MARCKS) was partially purified from bovine brain by the method of Graff et al. (14). Bovine myelin basic protein (MBP), potato acid phosphatase, and Staphylococcus aureus V8 protease were purchased from Sigma. [γ-32P]ATP (300 Ci/mmoll; 1 Ci = 37 GBq) was obtained from New England Nuclear. Phosphatidylserine (PtdSer), diolein (DO), and arachidonic acid were purchased from Serdary Research Laboratories (London, ON, Canada). Phorbol 12-myristate 13-acetate (PMA) and mezerein were the products of LC Services (Woburn, MA). Teleocidin was from Funakoshi (Tokyo). Leupeptin was a product of Peptide Institute (Osaka). Okadaic acid and (p-amidinophenyl)methanesulfonyl fluoride hydrochloride (p-APMSF) were purchased from Wako Pure Chemical (Osaka). Staurosporine and K-252b were the products of Kyowa Medex (Tokyo). Other chemicals were from commercial sources.

Enzyme and Assay. αPKC was assayed and purified to homogeneity from rat brain cytosol as described (15). δPKC activity was routinely assayed by measuring the incorporation of 32P into MBP from [γ-32P]ATP. The standard reaction mixture (50 μl) contained 20 mM Tris/HCl (pH 7.5), 10 mM MgCl2, 10 μM [γ-32P]ATP (2 × 106 cpmp/μmol), MBP (200 μg/ml), PtdSer (16 μg/ml), DO (1.6 μg/ml), 0.5 mM EGTA, and the enzyme. Basal activity was measured without PtdSer and DO. After incubation for 10 min at 30°C, the reaction was terminated by spotting an aliquot of the mixture onto P81 paper (Whatman). The paper was washed five times, 5 min each, by immersion in 10 ml of 75 mM H3PO4. The radioactivity remaining on the paper was quantitated by Cerenkov counting. One unit of PKC was defined as the amount of enzyme that incorporated 1 nmol of phosphate per min into MBP under standard conditions.

Transfection of δPKC cDNA. The COS 7 cells expressing recombinant δPKC were prepared by transfecting δPKC cDNA-constructed plasmid (pTB801) as described (7).

V8 Protease Peptide Mapping. The purified 76-kDa enzyme (0.1 μg) from brain and COS 7 cells was autophosphorylated with [γ-32P]ATP (8 × 106 cpmp/μmol) in the presence of PtdSer (16 μg/ml), DO (1.6 μg/ml), and EGTA (0.5 mM). The reaction was terminated by Laemmli’s SDS/PAGE sample buffer, followed by heating for 3 min at 95°C. The radioactive

Abbreviations: PP2A, protein phosphatase 2A; MBP, myelin basic protein; DO, diolein; PtdSer, phosphatidylserine; p-APMSF, (p-amidinophenyl)methanesulfonyl fluoride hydrochloride; PMA, phorbol 12-myristate 13-acetate; PKC, protein kinase C; MARCKS, myristoylated alanine-rich C-kinase substrate.
enzyme was digested by various concentrations of *S. aureus* V8 protease for 30 min at 25°C. The digests were subjected to SDS/PAGE followed by autoradiography to visualize the 32P-labeled peptides.

**Phosphatase Treatment.** The enzyme fraction containing doublet proteins (76 and 78 kDa) was treated with the catalytic subunit of PP2A or potato acid phosphatase. Treatment of the enzyme with PP2A (75 units/ml) was carried out in 5 mM Tris·HCl (pH 7.5) containing 2.5 mM dithiothreitol, 0.25 mM EDTA, and 2.5% glycerol at 30°C as indicated. Control incubation was in the presence of 4 µM okadaic acid. Acid phosphatase (64 ng/ml) treatment was conducted in 100 mM 2-morpholinoethanesulfonic acid (Mes) (pH 5.5) containing 10 mM MgCl2 at 37°C as indicated. The reaction was stopped by Laemmli's SDS/PAGE sample buffer. After electrophoresis, the enzyme was subjected to immunoblot analysis with antibody CKpV38-a.

**Immunoblot Analysis.** Polyclonal antibodies were raised against synthetic oligopeptides that are parts of the deduced amino acid sequence of rat brain δPKC. The antibodies designated CKpV36-a and CKpV58-a were prepared against the synthetic oligopeptides Lys-Pro-Glu-Thr-Pro-Glu-Thr-Val-Gly-Ile-Tyr-Gln-Gly-Lys and Ser-Phe-Ser-Asp-Lys-Asn-Leu-Ile-Asp-Ser-Met-Asp-Gln-Thr-Ala, which are parts of the amino acid sequence corresponding to the sequences 301–317 and 643–657 of the δ-subspecies, respectively. Immunoblot analysis was carried out as described (8).

**Other Procedure.** Protein concentration was determined by the method of Bradford (16) with bovine serum albumin as a standard.

**RESULTS**

**Purification of δPKC.** Consistent with the Northern blot analysis described (7), Western blot analysis suggests that δPKC was abundant in brain tissues but was also expressed in many other tissues such as lung, spleen, and kidney. In the rat brain, although a small portion of δPKC was found in the cytosol, a large portion of this enzyme was normally associated with particulate fractions. Thus, the enzyme was extracted from rat brain particulate fractions by Triton X-100 before being purified. All manipulations were carried out at 0°C–4°C. The rat brain (~40 g wet weight) was homogenized in a Potter–Elvehjem Teflon glass homogenizer with 160 ml of 20 mM Mes (pH 6.5) containing 10 mM EGTA, 2 mM EDTA, 0.25 M sucrose, 20 mM 2-mercaptoethanol, leupeptin (100 µg/ml), and 50 µM p-APMSF (homogenizing buffer). After centrifugation for 60 min at 100,000 × g, the precipitate was suspended in 200 ml of the homogenizing buffer containing 1% (wt/vol) Triton X-100 and stirred for 1 hr. After centrifugation as described above, the supernatant was used as an enzyme source.

The Triton X-100 extract was applied to an S-Sepharose fast flow (Pharmacia; 3 × 10 cm) column equilibrated with buffer A, which contained 20 mM Mes (pH 6.5), 0.5 mM EGTA, 0.5 mM EDTA, 20 mM 2-mercaptoethanol, leupeptin (20 µg/ml), and 50 µM p-APMSF. The column was washed with 700 ml of buffer A containing 20 mM NaCl. The enzyme was eluted batchwise with 280 ml of buffer A containing 400 mM NaCl. The PKC activity dependent on PtdSer and DO could not be detected in this eluate. For further purification, all columns were equipped with an FPLC system (Pharmacia). The eluate was brought to 1 M NaCl (pH 7.5) by adding solid NaCl and 1 M Tris·HCl (pH 9.5). This enzyme solution was applied to a TSK phenyl 5PW [Toyoda Soda (Tokyo); 2.15 × 15 cm] column equilibrated with 1 M NaCl in buffer B, which contained 20 mM Tris·HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 20 mM 2-mercaptoethanol, leupeptin (20 µg/ml), and 50 µM p-APMSF. After washing with 250 ml of buffer B containing 1 M NaCl, PKC was eluted from the column by application of a 360-ml decreasing linear concentration gradient of NaCl (1.0–0 M) in buffer B at a flow rate of 4 ml/min. Fractions (7 ml each) were collected and assayed for enzyme activity. A broad peak appeared, which was not absolutely dependent on PtdSer and DO. However, immunoblot analysis of each fraction showed a peak that was immunoreactive with antibodies against δPKC synthetic peptide (Fig. 1A).

The fractions of this immunoreactive peak (fractions 30–39), which also contained several other PKC subspecies, were pooled, diluted with 140 ml of buffer B, and loaded onto

![Fig. 1.](image-url) Elution profile of δPKC from rat brain and COS 7 cells transfected with δPKC cDNA. PKC activity was assayed with MBP as a substrate under standard conditions. •, in the presence of PtdSer, DO, and EGTA; ◦, in the presence of EGTA alone. (Insets) Immunoblot of each fraction with antibody CKpV36-a. (A) Rat brain enzyme from phenyl 5PW. (B) Rat brain enzyme from heparin 5PW. (C) Rat brain enzyme from hydroxyapatite. (D) COS 7 cell enzyme from hydroxyapatite.
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Fig. 2. Separation of 76- and 78-kDa δPKC on a Mono Q column. (A) Elution profile of δPKC from a Mono Q column. δPKC activity was assayed with MBP as a substrate under standard conditions. ↓, In the presence of PtdSer, DO, and EGTA; ○, in the presence of EGTA alone. (B) Coomassie blue staining. (C) Immunoblot analysis. Aliquots from Mono Q fractions were analyzed by SDS/PAGE and immunoblot analysis with antibody CKpV38-a.

A TSK heparin 5PW [Toyo Soda (Tokyo); 2.15 × 15 cm] equilibrated with buffer B. After washing with 250 ml of buffer B containing 20 mM NaCl, the enzyme was eluted by application of a 360-ml linear concentration gradient of NaCl (0–0.35 M) in buffer B at a flow rate of 4 ml/min. Fractions (6 ml each) were collected. Two enzyme peaks, both of which were dependent on PtdSer and DO, were eluted at 0.14 and 0.26 M NaCl (Fig. 1B). The first peak was reactive with antibody CKpV38-a (Fig. 1B Inset). The identification of the second peak remains to be clarified. δPKC was not separated from the other subspecies of PKC until this step of purification.

Fractions 22–25 were pooled and subjected directly on a packed column of hydroxyapatite [Koken (Tokyo); 0.78 × 10 cm] equilibrated with buffer C, which contained 20 mM potassium phosphate (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10% (vol/vol) glycerol, 20 mM 2-mercaptoethanol, leupeptin (20 μg/ml), 50 μM P-APMSF, and 0.02% Triton X-100. The column was washed with 15 ml of buffer C at a flow rate of 0.4 ml/min. δPKC was then eluted by application of an 84-ml linear concentration gradient of potassium phosphate (20–250 mM) in buffer C. Fractions (1 ml each) were collected. The elution profile of enzyme activity coincided with that of immunoreactivity (Fig. 1C). δPKC appeared between βPKC and αPKC on the hydroxyapatite column when cochromatographed.

To purify the enzyme further, the active fractions (fractions 37–41) were pooled, dialyzed against buffer B, and then applied onto a Mono Q HR5/5 column (Pharmacia; 0.5 × 5 cm) equilibrated with buffer B. After washing with 20 ml of buffer B at a flow rate of 0.5 ml/min, δPKC was eluted by application of a 50-ml linear concentration gradient of NaCl (0–0.35 M) in buffer B. Fractions (1 ml each) were collected. δPKC activity could be separated partially into two peaks with a small shoulder (Fig. 2A). Coomassie staining of the SDS/polyacrylamide gel (Fig. 2B) and immunoblot with antibody CKpV38-a (Fig. 2C) revealed that δPKC exists in two forms. The molecular masses of the first and second peaks were estimated to be 76 and 78 kDa, respectively. Both enzymes were also recognized by antibody CKpV58-a raised against the C-terminal oligopeptide of δPKC. The purification procedures are summarized in Table 1.

δPKC from COS 7 Cells. The recombinant δPKC expressed in COS 7 cells, which were transfected by δPKC cDNA-constructed plasmid, was purified for comparison. Three days after transfection, the COS 7 cells (4 × 10⁶ cells) were harvested and homogenized by sonication with a Kontes sonifier for 1 min in 3 ml of the homogenizing buffer containing 1% Triton X-100. After stirring for 30 min on ice, the homogenate was centrifuged for 60 min at 100,000 × g. The supernatant was applied to an S-Sepharose fast flow column (0.25 × 2 cm) equilibrated with buffer A. After washing the column with 10 ml of buffer A containing 20 mM NaCl, the enzyme was eluted batchwise with 4 ml of buffer A containing 400 mM NaCl. After the eluate was adjusted to pH 7.5 by adding 1 M Tris-HCl (pH 9.5), the enzyme was applied to a hydroxyapatite column (0.78 × 10 cm) and eluted as described above. The recombinant PKC was eluted at the same potassium phosphate concentration as the endogenous δPKC obtained from brain tissue (Fig. 1D). When subjected to immunoblot analysis with CKpV38-a, the enzyme again showed doublets proteins of 76 and 78 kDa.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Vol, ml</th>
<th>Protein, mg</th>
<th>Activity, units</th>
<th>Specific activity, units/mg</th>
<th>Yield, %</th>
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<tr>
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<tr>
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<td>—</td>
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<td>177.8</td>
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* These values do not represent solely the activity of δPKC but include the activity of some other PKC subspecies.
Phosphatase Treatment. When the fractions containing the doublet proteins obtained from the rat brain and COS 7 cells were treated with the catalytic subunit of PP2A, the 76-kDa protein decreased with the concomitant increase of the 78-kDa protein (Fig. 3 A and B). This shift of mobility was inhibited by okadaic acid, a specific inhibitor of PP2A. When potato acid phosphatase was used instead of PP2A, the 78-kDa protein disappeared (Fig. 3 C and D). The results suggest that OPKC exists in two forms—78-kDa phosphorylated and 76-kDa nonphosphorylated—in both the brain and COS 7 cells. When the 76-kDa enzyme was autophosphorylated, the enzyme did not migrate to the 78-kDa enzyme, suggesting that two forms of OPKC do not result from autoprophosphorylation. These two forms were indistinguishable from each other in their specific activity; response to PtdSer, DO, and Ca2+.

Peptide Mapping. Both purified enzymes from the rat brain and COS 7 cells were autophosphorylated with [γ-32P]ATP in the presence of PtdSer and DO and treated with S. aureus V8 protease. The resulting radioactive peptides were then subjected to SDS/PAGE and visualized. Phosphopeptide mapping was the same for the enzymes from the two sources (Fig. 4), providing additional evidence that OPKC purified from the rat brain is the product of the OPKC gene.

Kinetic Properties. In contrast to αPKC, OPKC from the rat brain as well as from COS 7 cells was independent of Ca2+ concentration for its activity (Fig. 5). In addition to PtdSer, either DO or PMA was absolutely required for phosphorylation of MBP. Other tumor promoters, such as mezerein and teleocidin, could activate OPKC in the presence of PtdSer. Recently, free cis-unsaturated fatty acids such as arachidonic and oleic acids were shown to act synergistically with DO for activation of α-, β-, and γPKC (17). For OPKC, however, arachidonic acid could not synergize with PtdSer and DO but rather was inhibitory (Fig. 6). OPKC was more resistant than αPKC to staurosporine and K-252b, which are known powerful inhibitors of PKC. Although αPKC was inhibited 70% by K-252b (100 nM), OPKC was totally insensitive at the same concentration.

Substrate Specificity. OPKC could phosphorylate various synthetic peptide substrates, such as MBP<sub>1-14</sub> (Gln-Lys-Arg-Pro-Ser-Gln-Arg-Ser-Lys-Tyr-Leu), an optimal growth factor receptor (EGFR<sub>638-648</sub> (Val-Arg-Lys-Arg-Thr-Leu-Arg-Arg-Leu)), and S623-239 (Arg-Leu-Ser-Arg-Ala), all of which are preferable substrates for α-, β-, and γPKC (18).
MARCKS has been shown to be a prominent physiological substrate for PKC in a variety of cells and has been implicated in control of diverse cellular functions (14). PKC phosphorylated MARCKS significantly, and this reaction was again insensitive to Ca\(^{2+}\) but absolutely required DO.

**DISCUSSION**

The \(\delta\)-subspecies of PKC was first isolated by molecular cloning from the rat brain cDNA library (7). The enzyme was subsequently expressed transiently in COS 7 cells by transfecting this cDNA-containing plasmid and was partially purified (7). An enzyme that is reactive with antibodies raised against a synthetic oligopeptide having a part of the predicted amino acid sequence has recently been partially purified from porcine spleen (13). The structural and genetic identity of the \(\delta\)-subspecies from mammalian tissues, however, has not yet been fully defined, although the \(\alpha-, \beta-, \gamma-, \) and \(\gamma(L)\)-subspecies have been well characterized (15). One of the difficulties of isolating the members of the second PKC subgroup, the \(\delta-, e-, \zeta-, \) and \(\eta(L)\)-subspecies is probably due to their apparently low activities with H1 histone as substrate. The \(\delta\)-subspecies isolated from the rat brain particulate fraction and the enzyme artificially expressed in COS 7 cells as described above show identical physical and kinetic properties. Both enzymes reveal doublet proteins upon gel electrophoresis, one of which is a phosphorylated form of the other. The nature of protein kinase responsible for its phosphorylation remains unknown at the present. The elution position of the \(\delta\)-subspecies on a hydroxyapatite column is between the \(\beta\)- and \(\alpha\)-subspecies. The purification and elution positions of other members of the second PKC subgroup will be described in subsequent papers.

As predicted (7), the \(\delta\)-subspecies is not sensitive to the Ca\(^{2+}\) concentration when histone, MBP, and MARCKS are used as phosphate acceptors. However, the enzyme is absolutely dependent on diacylglycerol. It has been recently reported that diacylglycerol may also be produced from phosphatidylincholine at a relatively later phase in cellular responses when cells are stimulated by long-acting signals such as some growth factors (for review, see ref. 19). In addition, it becomes plausible that sustained activation of PKC is essential for long-term cellular responses such as cell proliferation and differentiation (20, 21). In fact, it has been reported that PKC activity is increased by the action of some growth factors (for review, see ref. 22). It is an attractive idea to investigate whether the second subgroup of PKC, including the \(\delta\)-subspecies described in this paper, may take part in the protein kinase cascade reaction starting from the receptor stimulation eventually leading to control of the cell cycle.

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