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Biochemistry

Protein kinase C ζ subspecies from rat brain: Its structure, expression, and properties

(cDNA/gene expression)

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ABSTRACT **The primary structure of the ζ subspecies of rat brain protein kinase C was deduced from its overlapping cDNAs. The ζ subspecies of protein kinase C consists of 592 amino acid residues with the calculated molecular mass of 67,740 Da and has regulatory and protein kinase domains in its amino- and carboxyl-terminal halves, respectively. Although all members of the protein kinase C family so far identified have a tandem repeat of the characteristic cysteine-rich zinc-finger-like sequence in the regulatory domain, the ζ subspecies contains only one set of this sequence. Northern (RNA)-blot hybridization analysis indicated that two major RNA transcripts of the ζ subspecies with different lengths may be generated by the use of different polyadenylylational signals. The enzyme was expressed in COS-7 cells by transfection with the cDNA construct encoding its whole sequence. It showed an approximate molecular mass of 64,000 Da upon SDS/PAGE. The enzyme activity was significantly dependent on phospholipid but was independent of the presence of Ca^{2+} or diacylglycerol, when assayed with calf thymus H1 histone as a phosphate acceptor protein. The ζ subspecies expressed in COS-7 cells did not appear to show binding activity of phorbol ester. The structural and biochemical properties indicate that the ζ subspecies is related to, but distinct from, other subspecies of protein kinase C. Perhaps, this subspecies belongs to another entity of the enzyme family.**

The physiological importance of protein kinase (PKC) is now widely accepted and well documented (1). Molecular cloning and biochemical analysis has revealed the enzyme to exist as a family of multiple subspecies having closely related structures (1). Initially, four cDNA clones, α, βI, βII, and γ were isolated (2–8). The four PKC subspecies all consist of a single polypeptide with four conserved (C_1–C_4) and five variable (V_1–V_5) regions. The amino-terminal half, containing regions C_1 and C_2, is presumably the regulatory domain that interacts with Ca^{2+}, phospholipid, and diacylglycerol or phorbol ester, whereas the carboxyl-terminal half, containing regions C_3 and C_4, appears to be the protein kinase domain, as it shows large clusters of sequences that resemble many other protein kinases. The region C_1 contains a tandem repeat of the characteristic cysteine-rich zinc-finger-like sequence. The structure and genetic identity of these subspecies have been determined by comparison with the enzymes that are separately expressed in mammalian COS-7 cells transfected by the respective cDNA-containing plasmids (9, 10) and by immunoblot analysis, using type-specific antibodies, of COS-7 cells transfected with plasmids containing cDNA inserts of the different PKC subspecies (11). Recently, several additional cDNA clones designated δ, ε, and ζ, were isolated from a rat brain library by using a mixture of α, βII, and γ cDNAs as probes under low-stringency conditions (12, 13). A cDNA clone designated nPKC, probably encoding the ε subspecies, was also found in a rabbit brain cDNA library (14). Another cDNA clone, RP16, isolated previously from a rat brain library (15) may also encode a part of the ε subspecies. The three PKC molecules have a common structure closely related to, but clearly distinct from, the four subspecies initially described. The enzyme encoded by δ, ε, and ζ cDNA all lack the region C_2, and the translational products of δ and ε cDNA in COS-7 cells did not show an absolute requirement of Ca^{2+}, phospholipid, and diacylglycerol (13). On the other hand, the structure and enzymatic properties of the ζ-subspecies remain unknown, because the full length of its cDNA has not been available. This paper will describe the complete structure, expression, and some kinetic properties of the ζ subspecies of PKC.‡

MATERIALS AND METHODS

Isolation and Characterization of cDNA Clones. Two cDNA clones, λCKRLζ5 and λCKRLζ8, both encoding the ζ subspecies of PKC were isolated from a rat brain cDNA library, which was constructed in λgt10, by using a 0.4-kilobase (kb) *Eco*RI/*Cla* I restriction fragment from the 5′-terminal portion of λCKRζ3 insert (13) as a hybridization probe (Fig. 1). Plaque screening was carried out under the high-stringency condition described (13). The nucleotide sequence of the cDNA inserts was determined by using the deoxynucleotide chain-termination method by subcloning suitable overlapping restriction fragments into M13 series phage DNA, followed by primed DNA synthesis on single-stranded DNA templates in the presence of dideoxynucleotide triphosphates (16).

Northern (RNA) Blot Analysis. Northern blot analysis was carried out as described (13). Total RNAs prepared from the rat brain were denatured with 2.2 M formaldehyde and 50% (vol/vol) formamide, electrophoresed on a 1% agarose gel containing 2.2 M formaldehyde, and then transferred to a nitrocellulose filter. The RNAs were hybridized with the nick-translated ^{32}P-labeled probe at 42°C in 50% formamide containing 5× SSPE (1× SSPE = 180 mM NaCl/10 mM sodium phosphate at pH 7.4/1 mM EDTA), 5× Denhardt's solution (1× Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin), and 0.1% SDS/sonicated-denatured salmon sperm DNA at 100 μg/ml. The filter was washed at 55°C in 0.1× SSC (1× SSC = 150 mM NaCl/15 mM sodium citrate at pH 7.0) containing

Abbreviations: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; PBt_2, phorbol 12,13-dibutyrate; nt, nucleotide(s); C and V, conserved and variable regions, respectively.

‡The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg (accession no. J04532).

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0.1% SDS. The dried filter was exposed to an x-ray film for 2 days at −70°C with an intensifying screen. Sizes of the RNAs were determined with RNA markers (Bethesda Research Laboratories).

Construction of Expression Plasmid. The 0.85-kb *Eco*RI/*Cla* I DNA fragment from λCKRLζ8 and 1.43-kb *Cla* I/*Eco*RI DNA fragment from λCKRζ3 were prepared. These two fragments were assembled and introduced into the *Eco*RI site of an expression vector of pTB701 (13). The resultant plasmid is referred to as pTB949.

Transfection to COS-7 Cells. Fresh monolayers of COS-7 cells, in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, were transfected with each plasmid DNA by the calcium phosphate coprecipitation technique (17). After 3.5 hr of transfection, the cells were shocked with glycerol for 3 min at room temperature (18), and fresh medium was added to each plate. After 18 hr at 37°C the medium was replaced by fresh Dulbecco's modified Eagle's medium containing 5% fetal calf serum. Cells were harvested after an additional 48-hr incubation at 37°C.

Purification and Assay of Enzyme. The COS-7 cells (3×10^7 cells) transfected with each plasmid DNA were homogenized by sonication with a Kontes sonicator for 1 min in 1.0 ml of 20 mM Tris·HCl at pH 7.5/0.25 M sucrose/10 mM EGTA/2 mM EDTA/leupeptin at 20 μg/ml. The homogenate was centrifuged for 60 min at 100,000 × *g*. The supernatant was diluted with 3 vol of buffer A (20 mM Tris·HCl at pH 7.5/0.5 mM EGTA/0.5 mM EDTA/10 mM 2-mercaptoethanol) and applied to a TSK DEAE-5PW column (0.75 × 7.5 cm, Toyo Soda, Tokyo), which was connected to a Pharmacia FPLC system and equilibrated with buffer A. PKC was eluted by application of a 22.5-ml linear concentration gradient of NaCl (0–0.6 M) in buffer A. Fractions of 0.75 ml each were collected. All procedures described above were carried out at 0–4°C. PKC was assayed by measuring the incorporation of $^{32}P_i$ into calf thymus H1 histone from [γ-^{32}P]ATP in the presence of phosphatidylserine at 8 μg/ml, diolein at 0.8 μg/ml, or phorbol 12-myristate 13-acetate (PMA) at 16 ng/ml and various concentrations of $CaCl_2$ under the conditions specified earlier (10, 11).

Preparation of Antiserum. The antiserum designated CKpV5ζ-a was raised as described (19) against a synthetic oligopeptide, Gly-Phe-Glu-Tyr-Ile-Asn-Pro-Leu-Leu-Leu-Ser-Ala-Glu-Glu-Ser-Val. This sequence appears in the predicted carboxyl-terminal-end region of the ζ subspecies (amino acid residues 577–592, see below). The oligopeptide (0.8 mg) was coupled to bovine thyroglobulin (1 mg), emulsified in Freund's complete adjuvant, and injected s.c. into a New Zealand White rabbit. Three subsequent immunizations were carried out at 2-week intervals with the same amount of antigen in Freund's incomplete adjuvant. One week after the final immunization, the rabbit was bled, and the antiserum was purified by affinity chromatography on a column of anti-rabbit IgG-coupled Sepharose.

Immunoblot Analysis. PKC fractions were subjected to 8% SDS/PAGE (20) and transferred to a nitrocellulose filter (21). The filter was incubated overnight at room temperature with Tris·NaCl buffer (10 mM Tris·HCl at pH 7.5/150 mM NaCl) containing 3% (wt/vol) gelatin and 0.02% NaN_3 and then incubated with antiserum CKpV5ζ-a (≈5 μg/ml in Tris·NaCl buffer containing 5% normal goat serum) for 1 hr at room temperature. After being washed with Tris·NaCl buffer containing 0.05% Tween 20, the filter was incubated for 30 min with biotinylated anti-rabbit IgG and subsequently with avidin-biotinylated horseradish peroxidase complex (Vectastain, Vector Laboratories). The color reaction used diaminobenzidine tetrahydrochloride and H_2O_2. Molecular masses were determined with biotinylated standard proteins (Bio-Rad).

Assay of Phorbol Ester Binding. Phorbol ester binding to intact COS-7 cells was analyzed as described (14). COS-7 cells (5×10^5 cells per 6-cm plate) were transfected with each plasmid DNA as described above. Three days after transfection, the cells were washed twice with binding solution (Dulbecco's modified Eagle's medium containing bovine serum albumin at 1 mg/ml and 10 mM Hepes at pH 7.0), and 1 ml of [^{3}H]phorbol 12,13-dibutyrate (PBt_2) (5 nM, Amersham 14.3 Ci/mmol; 1 Ci = 37 GBq) in the binding solution was added to the individual plates. After incubation for 30 min at 37°C, the cells were washed three times with ice-cold phosphate-buffered saline and harvested to measure the radioactivity and cell numbers.

RESULTS

Structure of ζ Subspecies. The cDNA clone λCKRζ3, which was previously reported (12, 13), does not contain a full length of the coding region of the ζ subspecies, because the open reading frame continued to the 5′-terminal direction, and a potential initiation codon of ATG is not found in the terminal region. Thus, rescreening for cDNAs that contain the amino-terminal-end coding region of this subspecies was done with a 5′-terminal restriction fragment of the λCKRζ3 insert as probe. From the rat brain cDNA library, two cDNA clones, designated λCKRLζ5 and λCKRLζ8, were isolated. Fig. 1 summarizes the series of the overlapping clones. The nucleotide sequence analysis of these cDNA inserts revealed the presence of an open reading frame that covers the entire coding region of the ζ subspecies. Fig. 2 shows the nucleotide and deduced amino acid sequences of the ζ subspecies. The open reading frame starts with a potential initiation codon of ATG [nucleotides (nt) 148–150]. This codon is flanked by sequences that fulfill the Kozak's criteria for initiation (22). Stop codon of TGA is found at nt 124–126 in-frame upstream from the initiation codon and at nt 1924–1926. The open reading frame encodes 592 amino acids with the molecular mass of 67,740 Da. The nucleotide sequence of the 5′-terminal region of λCKRζ3 was different from those of λCKRLζ5 and λCKRLζ8. The sequence of the divergent point of λCKRζ3 insert, ---CCGAG/(nt 32–36 of λCKRζ3, see ref. 13), agrees with the consensus sequence of the acceptor site of splicing (23). It is likely, therefore, that λCKRζ3 is derived from an unspliced molecule and that the 5′-terminal sequence of λCKRζ3 insert (nt 1–36, see ref. 13) is encoded by the sequence of an intron. It is particularly worth noting that the ζ subspecies of PKC contains only one set of the characteristic cysteine-rich zinc-finger-like sequence in the region C_1, whereas all other subspecies so far identified contain a tandem repeat of the sequence.

Northern blot (RNA) hybridization analysis using a restriction fragment of λCKRζ3 has indicated that two major RNA bands of 2.2 and 4.2 kb are present in some rat tissues, such as brain, kidney, and lung (13). The two RNA transcripts of different lengths were detected in rat brain RNAs when a

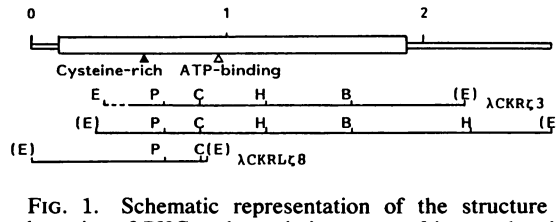

FIG. 1. Schematic representation of the structure of the ζ subspecies of PKC and restriction maps of its overlapping cDNA clones. The coding and noncoding sequences are indicated by thick and thin boxes, respectively. The cysteine-rich zinc-finger-like sequence and ATP-binding site are indicated by closed and open triangles, respectively. The restriction maps of λCKRζ3, λCKRLζ5, and λCKRLζ8 are shown by lines. B, *Bam*H1; C, *Cla* I; E, *Eco*RI; H, *Hin*dIII; and P, *Pst* I. The *Eco*RI sites in parentheses are derived from the *Eco*RI linkers ligated to the cDNAs during the cloning procedures. Scale given at top of figure is in kb of nt.

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CGAGGCGGGTGCCGTCGGTCCCGCGCTGCGCGCTCCCTTCCGCGTTCCGCCGTCTGCCCCGCCTGGCAACCCGGCCCTGCTCGGGGGCCGCTAGCCATGGCCGGAGTGCCCTGGACACAG  120

CGCTGACGGCGGCTGGCGGAGTGCGCCATGCCCAGCAGGACCGACCCCAAGATGGACCGGAGCGGCGGCCGCGTCCGTCTGAAGGCGCACTACGGCGGGGACATCCTGATTACCAGCGTG  240
    *                    M  P  S  R  T  D  P  K  M  D  R  S  G  G  R  V  R  L  K  A  H  Y  G  G  D  I  L  I  T  S  V   31

GACCCCACGACAACTTTCCAGGACCTCTGTGAGGAAGTGCGAGACATGTGTGGCCTGCACCAGCAGCACCCACTCACCCTCAAGTGGGTGGACAGTGAAGGTGACCCTTGTACTGTGTCC  360
 D  P  T  T  T  F  Q  D  L  C  E  E  V  R  D  M  C  G  L  H  Q  Q  H  P  L  T  L  K  W  V  D  S  E  G  D  P  C  T  V  S   71

TCACAGATGGAGCTGGAGGAGGCCTTCCGCCTGGCCTGTCAGGGCAGGGACGAAGTGCTCATCATCCACGTTTTCCCAAGCATCCCAGAACAACCGGGCATGCCTTGTCCTGGAGAAGAC  480
 S  Q  M  E  L  E  E  A  F  R  L  A  C  Q  G  R  D  E  V  L  I  I  H  V  F  P  S  I  P  E  Q  P  G  M  P  C  P  G  E  D  111
 ▼
AAGTCCATCTACCGCCGTGGAGCCAGAAGATGGAGGAAGCTATACCGAGCCAACGGCCACCTCTTCCAAGCCAAGCGCTTTAACAGGAGAGCGTACTGTGGCCAGTGCAGCGAAAGGATA  600
 K  S  I  Y  R  R  G  A  R  R  W  R  K  L  Y  R  A  N  G  H  L  F  Q  A  K  R  F  N  R  R  A  Y  C  G  Q  C  S  E  R  I  151
                                                                                 ●              ●
TGGGGCCTCGCGAGGCAGGGGTACAGGTGCATCAACTGCAAGCTGCTTGTCCATAAACGCTGCCACGTCCTCGTCCCGCTGACCTGCAGGAGGCATATGGATTCTGTCATGCCTTCCCAA  720
 W  G  L  A  R  Q  G  Y  R  C  I  N  C  K  L  L  V  H  K  R  C  H  V  L  V  P  L  T  C  R  R  H  M  D  S  V  M  P  S  Q  191
                           ●        ●              ●                          ●
GAGCCTCCAGTAGATGACAAGAACGATGGTGTAGACCTTCCTTCAGAAGAAACTGATGGAATTGCTTATATTTCTTCATCTCGGAAACATGACAATATCAAAGATGATTCTGAGGACCTT  840
 E  P  P  V  D  D  K  N  D  G  V  D  L  P  S  E  E  T  D  G  I  A  Y  I  S  S  S  R  K  H  D  N  I  K  D  D  S  E  D  L  231

AAGCCTGTCATCGATGGGGTGGATGGGATCAAAATCTCTCAGGGGCTGGGGCTGCAAGACTTTGACCTCATCAGAGTCATCGGGCGTGGAAGCTATGCCAAGGTCCTCCTGGTGCGGTTG  960
 K  P  V  I  D  G  V  D  G  I  K  I  S  Q  G  L  G  L  Q  D  F  D  L  I  R  V  I  G  R  G  S  Y  A  K  V  L  L  V  R  L  271

AAGAAAAACGACCAGATTTACGCCATGAAGGTGGTGAAGAAGGAGCTCGTCCACGACGATGAGGATATCGACTGGGTGCAGACAGAGAAGCACGTGTTCGAGCAGGCATCCAGCAACCCC 1080
 K  K  N  D  Q  I  Y  A  M  K  V  V  K  K  E  L  V  H  D  D  E  D  I  D  W  V  Q  T  E  K  H  V  F  E  Q  A  S  S  N  P  311

TTCCTGGTTGGCTTACACTCCTGCTTCCAGACAACGAGCCGGTTGTTCCTGGTCATCGAGTATGTCAACGGGGGGGACCTCATGTTCCACATGCAGAGGCAGAGGAAGCTTCCAGAGGAA 1200
 F  L  V  G  L  H  S  C  F  Q  T  T  S  R  L  F  L  V  I  E  Y  V  N  G  G  D  L  M  F  H  M  Q  R  Q  R  K  L  P  E  E  351

CACGCCAGGTTCTATGCTGCTGAGATCTGTATCGCTCTCAACTTCCTACATGAGAGAGGGATCATCTACCGGGACCTAAAACTGGACAACGTCCTCCTCGATGCCGATGGACACATTAAG 1320
 H  A  R  F  Y  A  A  E  I  C  I  A  L  N  F  L  H  E  R  G  I  I  Y  R  D  L  K  L  D  N  V  L  L  D  A  D  G  H  I  K  391

CTGACGGACTACGGCATGTGCAAGGAAGGCCTAGGCCCCGGCGACACAACAAGCACTTTTTGTGGAACCCCGAACTATATCGCCCCCGAAATCCTGCGAGGAGAAGAGTACGGGTTCAGC 1440
 L  T  D  Y  G  M  C  K  E  G  L  G  P  G  D  T  T  S  T  F  C  G  T  P  N  Y  I  A  P  E  I  L  R  G  E  E  Y  G  F  S  431

GTGGACTGGTGGGCGCTGGGTGTCCTTATGTTTGAGATGATGGCTGGGCGCTCCCCCTTTGACATCATCACAGACAACCCTGACATGAATACTGAAGACTACCTTTTCCAAGTTATCCTG 1560
 V  D  W  W  A  L  G  V  L  M  F  E  M  M  A  G  R  S  P  F  D  I  I  T  D  N  P  D  M  N  T  E  D  Y  L  F  Q  V  I  L  471

GAAAAGCCAATTCGGATTCCCCGTTTCCTGTCTGTCAAGGCCTCACACGTCTTGAAAGGATTTTTAAATAAGGATCCCAAAGAGAGGCTTGGCTGCCGGCCGCAGACTGGGTTTTCCGAC 1680
 E  K  P  I  R  I  P  R  F  L  S  V  K  A  S  H  V  L  K  G  F  L  N  K  D  P  K  E  R  L  G  C  R  P  Q  T  G  F  S  D  511

ATCAAGTCCCATGCCTTCTTCCGAAGCATAGACTGGGACCTGCTTGAAAAGAAGCAGACCCTGCCTCCCTTCCAGCCCCAGATCACAGATGACTATGGCCTGGACAACTTCGACACGCAG 1800
 I  K  S  H  A  F  F  R  S  I  D  W  D  L  L  E  K  K  Q  T  L  P  P  F  Q  P  Q  I  T  D  D  Y  G  L  D  N  F  D  T  Q  551

TTCACCAGCGAGCCCGTACAGCTGACCCCAGATGATGAGGACGTCATAAAGAGGATCGACCAGTCCGAGTTCGAAGGCTTCGAGTACATCAACCCGCTTCTGCTGTCTGCTGAGGAGTCC 1920
 F  T  S  E  P  V  Q  L  T  P  D  D  E  D  V  I  K  R  I  D  Q  S  E  F  E  G  F  E  Y  I  N  P  L  L  L  S  A  E  E  S  591

GTGTGAGGCCATGAGCATCTCTGTTGTGGACACGTCTGTGAATGACCCTGTCACTTTACCCTTAACTACAGCATATGCATGCCAGGCCAGACACCGAGGCTCCAAGCAGCCAGAGAGGGA 2040
 V  *                                                                                                                      592

TGCTGGCCACCAAGACCGCAGAGGGGGCACCCAACAGGCACTTCTAGACAGAGCAATCTCTTGTGTCCAGGCCCCAGAGGCTGGCTTTGTGCTGGAAGGAACCACTTCCTGTGCCCATGG 2160
CGGCCCTACCAGAGGGTGAGACAGCCACGCCGTCTTGAAAGGCGCACATCTTCCACAGAGACAGAACTCGATGCACTGATCCGCTCCAGGAAAAGTTAGCGTGTAATGCCCTGAGGAATA 2280
AAGTGTACTGATGATGTCGAAGCTTCCTCTGATGCCTTTTTTCTGTGGCACCCGCCTGATGTCCCCAATGCAGCCCCCCACACGAGGAGGAGCTGCGCTTTCCAAGCAGGCAGAACGTAG 2400
GCAGGGGACGGGCACCCAAGTGCCTGCAGGGATGTTGTGCTGCAACAAGAATTGTTACAGATTCGGAACGTTTTCTAAATCCCGGTAAGTTCTGTTGTTAATATTTAACAAGTATTTTCA 2520
TACCTAGGGCAGCTAACCGTGGTAGGCTGAAGCCGAAGGGACACCCCGGCCAGGTTGGTGGCGCTGTTCCCACCAGCCCAGCCCGTTCGGACTCTGAAGGGGGGGAAATTGGATCACGGA 2640
GATTCTAAAACATTGCTCACC                                                                                                    2661
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FIG. 2. DNA and deduced amino acid sequences of the ζ subspecies of PKC. Nucleotides and amino acids are numbered at the right. ⋆, Termination of coding region and first upstream in-frame stop codon in the 5′-untranslated sequence; ●, cysteine residues in the cysteine-rich zinc-finger-like sequence; ▼, divergent point of λCKRζ3, λCKRLζ5, and λCKRLζ8. Potential polyadenylylational signal is underlined. The single-letter amino acid code is used.

restriction fragment that encodes the 5′-untranslated and amino-terminal regions was used as a probe, whereas only the 4.2-kb transcript was found when the 3′-untranslated region downstream from the AATAAA sequence of a potential polyadenylylational signal (nt 2277–2282) was used as a probe (Fig. 3). This result suggests that there is another polyadenylylational signal in the 3′-portion from the AATAAA (nt 2277–2282), which is not included in the cDNAs obtained and that the two transcripts with different lengths may be generated by the use of the two polyadenylylational signals.

Expression of cDNA for ζ Subspecies. The cDNA for the ζ-subspecies was transiently expressed in mammalian COS-7 cells. The cDNA fragments were assembled and inserted into the vector of pTB701 to express the complete sequence of the ζ subspecies as described. Three days after transfection with the expression plasmid, pTB949, the cell extract was prepared and applied to a TSK DEAE-5PW column, which was connected to a HPLC system. As shown in Fig. 4, the protein kinase from the transfected cells was resolved into two major peaks (fractions 12–15 and fractions 17–21), whereas that of the untransfected control cells showed only one major peak, which eluted with a lower salt concentration at the position of the first peak. This major peak from the control COS-7 cells was previously shown to be the α subspecies of PKC (10, 11). The second peak from the transfected cells contained two proteins, which were reactive with an antibody that was prepared against the ζ subspecies as shown in Fig. 5. The protein having an approximate molecular mass of 64,000 Da appears to be the ζ subspecies, which should be 67,740 Da. The other immunoreactive protein having an approximate molecular mass of 30,000 Da was found to be associated with the shoulder of the second peak (fractions 19 and 20). It is possible that this small protein is a proteolytic fragment of the ζ subspecies, but its precise nature is not clear at present.

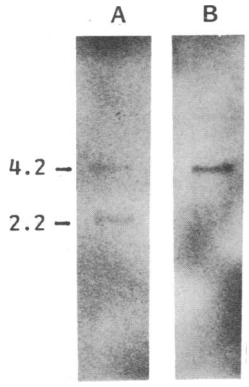

FIG. 3. Northern blot analysis of total RNAs from rat brain. The total RNAs were denatured, electrophoresed, transferred to nitrocellulose filters, and hybridized with the nick-translated ^{32}P-labeled probe for the ζ subspecies. (*A*) Northern blot analysis with a probe of the 5′ portion of cDNA of the ζ subspecies (nt 1–855, *Eco*RI/*Cla*I fragment of λCKRLζ8). (*B*) Northern blot analysis using a probe of the 3′ portion of cDNA of the ζ subspecies (nt 2300–2661, *Hin*dIII/*Eco*RI fragment of λCKRLζ5). Sizes of RNAs are given in kb.

Properties of ζ Subspecies. With calf thymus H1 histone as substrate, the ζ subspecies showed significant enzymatic activity without addition of Ca^{2+}, phospholipid, and diacylglycerol (Fig. 6*A*). This activity was enhanced by phospholipid. Neither diacylglycerol nor PMA showed any effect. These kinetic properties are in marked contrast to the α subspecies, an endogenous PKC in COS-7 cells (Fig. 6*B*).

The ζ subspecies did not bind phorbol ester both in the presence and absence of phospholipid, when tested with the partially purified enzyme preparations. The inability to bind phorbol ester was confirmed also with intact COS-7 cells, which express this PKC subspecies. The COS-7 cells trans-

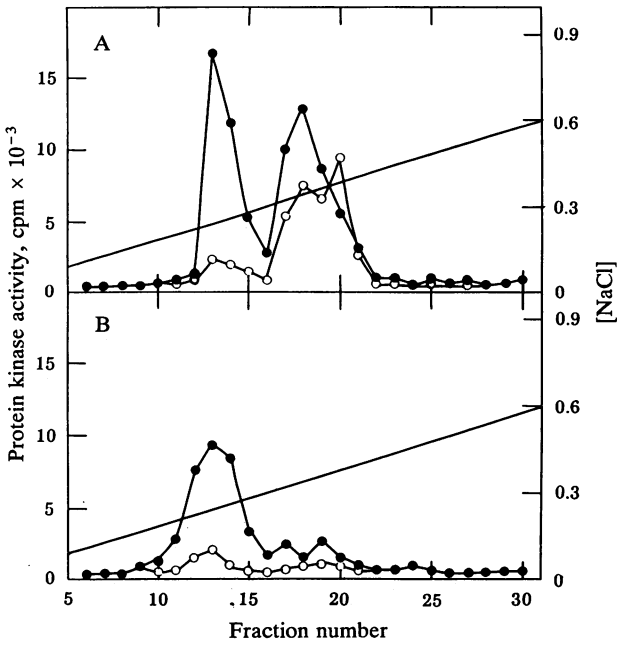

FIG. 4. Elution profile of protein kinases expressed in COS-7 cells on TSK DEAE-5PW column chromatography. The supernatants of the transfected and control COS-7 cells were applied to a TSK DEAE-5PW column, and PKC was eluted and assayed as described. (*A* and *B*) Protein kinases expressed in the pTB949 (ζ subspecies)-transfected COS-7 cells and untransfected control COS-7 cells, respectively. ●, Protein kinase activity with phosphatidylserine, diolein, and 0.5 mM $CaCl_2$; ○, protein kinase activity with 0.5 mM EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; and —, NaCl.

fected with pTB755, the α cDNA construct (6, 10), showed [^{3}H]PBt$_2$-binding activity 10-fold higher than that of the untransfected control COS-7 cells when tested with the intact cells. Under similar conditions, the COS-7 cells transfected with pTB949, the ζ-cDNA construct, did not show any increase in [^{3}H]PBt$_2$-binding activity, although these cells showed obvious protein kinase activity of the ζ subspecies as described above. The results are summarized in Table 1, indicating that the ζ subspecies may have, if any, weak phorbol ester binding activity.

DISCUSSION

The PKC subspecies encoded by α, βI, βII, and γ cDNAs have similar structures, and all consist of a single polypeptide

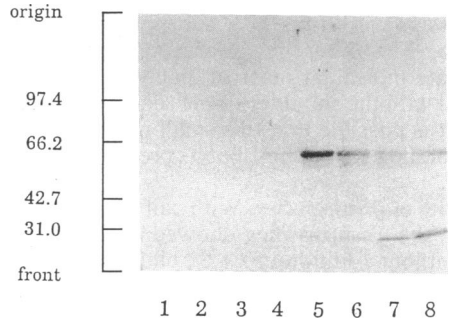

FIG. 5. Immunoblot analysis of the ζ subspecies of PKC. The TSK DEAE-5PW column fractions of pTB949-transfected cells were electrophoresed and transferred to a nitrocellulose filter, and immunoblot analysis was done as described. Lanes 1–8 correspond to fractions 13–20 in Fig. 4*A*. Molecular size standards used are shown in kDa: 97.4, phosphorylase *b*; 66.2, bovine serum albumin; 42.7, ovalbumin; and 31.0, chymotrypsinogen.

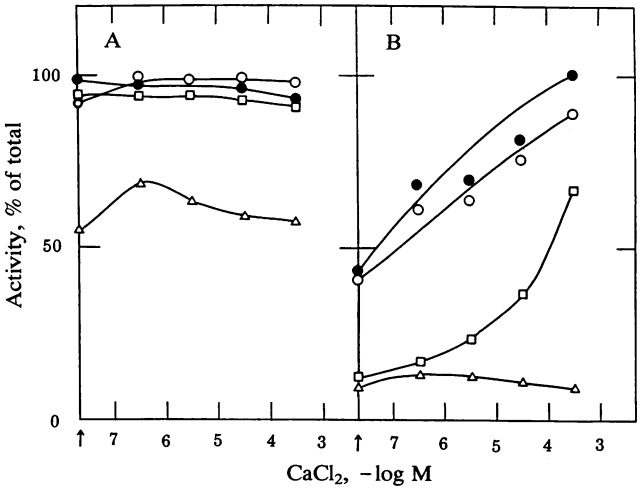

FIG. 6. Kinetic properties of protein kinases obtained from transfected COS-7 cells. The PKC fractions of TSK DEAE-5PW column chromatography were assayed in the presence of various concentrations of Ca^{2+}. (*A* and *B*) Protein kinase activity of fraction 18 (ζ subspecies) and fraction 13 (α subspecies), respectively, from the pTB949-transfected COS-7 cells given in Fig. 4*A*. Where indicated with an arrow, EGTA (1 mM at final concentration) was added instead of $CaCl_2$. ●, Protein kinase activity with phosphatidylserine and diolein; ○, protein kinase activity with phosphatidylserine and PMA; □, protein kinase activity with phosphatidylserine alone; △, protein kinase activity without phosphatidylserine, diolein, or PMA. Results were normalized to the maximum protein kinase activity that was obtained in the presence of $CaCl_2$, phosphatidylserine, and diolein or PMA.

with four conserved and five variable regions. These subspecies are activated by diacylglycerol or phorbol ester in the presence of phospholipid and Ca^{2+}, although their kinetic properties are subtly different from one another (1). The structure of the ζ subspecies, as δ and ε subspecies (13), is closely related to, but clearly distinct from those of α, βI, βII, and γ subspecies. The δ, ε, and ζ subspecies all lack the region C_2, and the three enzymes expressed in COS-7 cells do not show an absolute requirement of phospholipid and Ca^{2+}, implying a role of the region C_2 in interacting with Ca^{2+} and phospholipid (for δ and ε subspecies, see ref. 13). This enzyme property is reminiscent of the previously described rabbit reticulocyte protease-activated kinase II, which can phosphorylate the ribosomal S6 protein (24). The exact relation of this reticulocyte enzyme to the members of the PKC family thus far described remains unclear. However, it is important to note that the *in vitro* dependency of PKC on Ca^{2+}, phospholipid, and diacylglycerol varies markedly with the phosphate acceptor protein. A typical example is protamine, the phosphorylation of which by the PKC subspecies requires neither Ca^{2+}, phospholipid, nor diacylglycerol (25). In fact, kinetic properties of PKC have repeatedly been shown to vary greatly with the substrate used (26, 27), and it

Table 1. PBt$_2$-binding activity of the COS-7 cells transfected with plasmids

Sample	PBt$_2$-binding activity, cpm per 10^6 cells	
	Experiment 1	Experiment 2
Untransfected COS-7 cells	930	ND
pTB949 (ζ subspecies)	1010	670
pTB755 (α subspecies)	7220	9050
pTB701 (without insert)	940	790

COS-7 cells were transfected with each plasmid and PBt$_2$-binding activity of the cells was assayed as described. ND, not determined. Results of two independent transfection experiments are given.

is clear that comparison of the properties of the members of the PKC family is possible under only limited and defined conditions. Because each member of the family is apparently localized in very specialized intracellular compartments in specific cell types (1), each may phosphorylate distinctly different target proteins under physiological conditions. It is a general problem in protein phosphorylation research that in cell-free enzymatic reactions most protein kinases can phosphorylate many proteins, only some of which are physiologically significant. Thus, the possibility that the enzymes respond differently to Ca^{2+} and lipids *in vivo* greatly complicates the search for physiological substrates for members of the PKC family.

The four subspecies initially identified, α, βI, βII, and γ PKC, bind phorbol ester in the presence of Ca^{2+} and phospholipid. Although the nPKC from the rabbit brain, that corresponds to the ε subspecies, has been proposed to bind phorbol ester (14), no obvious reproducible PBt_2-binding activity has been demonstrated thus far for the ζ subspecies under the conditions specified above. Again, it has been pointed out that the PBt_2-binding to the PKC molecule varies greatly with the coexistence of substrate proteins (28). The members of the *raf* oncogene family encode a serine/threonine protein kinase domain in their carboxyl-terminal half and one set of a cysteine-rich zinc-finger-like sequence similar to those of the PKC family in their amino-terminal half (29–31). However, the amino acid sequence of the ζ subspecies is distinct from the products of the *raf* gene family such as c-*raf* and A-*raf*. Genomic Southern blot analysis with a DNA fragment from the ζ subspecies predicts the existence of some additional clones related to the ζ subspecies. Identification and characterization of further members of this enzyme family will provide additional information for the cellular signal pathways through the PKC family.

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