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# Expression of protein kinase C subspecies in rat retina

Naoko Fujisawa[a], Kouji Ogita[a], Naoaki Saito[c] and Yasutomi Nishizuka[a,b]

[a]*Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan,* [b]*Biosignal Research Center, Kobe University, Kobe 657, Japan* and [c]*Department of Pharmacology, Kobe University School of Medicine, Kobe 650, Japan*

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An extract of rat retina was subjected to Mono Q followed by chromatography on hydroxyapatite, and the protein kinase C (PKC) subspecies were identified by immunoblot and biochemical analysis. It was found that, although the relative activities assayed with myelin basic protein as a common phosphate acceptor vary greatly with one another, the α-, βI-, βII-, γ-, δ-, ε-, ζ-, and another structurally unknown PKC subspecies are expressed in this tissue. Thus, the retina is a unique tissue which expresses most of the PKC subspecies so far identified in mammals.

Protein kinase C; Retina

## 1. INTRODUCTION

Protein kinase C (PKC) appears to play crucial roles in cell surface signal transduction for the control of various physiological processes (for a review see [1]). Molecular cloning and biochemical analysis have revealed that, in mammalian tissues, PKC exists as a large family of at least eight subspecies (α, βI, βII, γ, δ, ε, ζ, and η) with closely related structures [1–3]. In the *Drosophila* visual system, a photoreceptor-specific PKC has been found, and its function in the light-dependent regulation of phototransduction has been postulated [4]. In fact, activators of PKC have been shown to decrease the light response and reduce the resting level as well as the light-induced decrease of cyclic GMP [5]. The presence of PKC in the outer segment of bovine retina [6] and its ability to phosphorylate rhodopsin [7] and arrestin [8] have been implicated in the functional significance of PKC in the visual transduction processes. Immunological analysis using specific antibodies against the α- and β-subspecies has shown their differential localization in the retinal neurons [9,10].

It has been well documented that, by hydroxyapatite column chromatography, the enzyme from brain tissues is resolved into three distinct fractions [11], which correspond to the enzyme subspecies encoded by γ, βI/βII and α PKC genes [12]. Recently, the enzyme subspecies encoded by δ and ε PKC genes have been purified and characterized from brain tissues [13,14]. To explore the function of PKC in the visual system, the subspecies of this enzyme family have to be characterized. The present studies were undertaken to identify the subspecies of PKC expressed in rat retinal tissue.

*Correspondence address:* N. Fujisawa, Department of Biochemistry, Kobe University School of Medicine, Kobe 650, Japan. Fax: (81) (78) 351-0082.

*Abbreviations:* PKC, protein kinase C; PtdSer, phosphatidylserine; MBP, myelin basic protein.

The data are taken in part from the dissertation that will be submitted by N. Fujisawa to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Sciences.

## 2. MATERIALS AND METHODS

### 2.1. *Materials and chemicals*

Bovine myelin basic protein (MBP) was purchased from Sigma. [γ-$^{32}$P]ATP (3,000 Ci/m mol) was obtained from New England Nuclear. Phosphatidylserine (PtdSer) and diolein were from Serdary Research Laboratories.

### 2.2. *Partial resolution of PKC subspecies*

All procedures were carried out at 0–4°C. Rat retina (680 mg wet weight) from thirty male Sprague-Dawley rats (4-week-old) was dissected and homogenized by sonication four times, each time for 5 s, in 4 ml 20 mM Tris-HCl pH 7.5, containing 10 mM EGTA, 2 mM EDTA, 20 mM 2-mercaptoethanol, 100 μg/ml leupeptin, 50 μM *p*-APMSF (*p*-amidinophenyl-methanesulfonyl fluoride hydrochloride) and 0.5% (v/v) Triton X-100. The homogenate was centrifuged for 60 min at 100,000 × *g*. The supernatant was adjusted to pH 8.0 by the addition of 1 M Tris-HCl pH 9.5, and then applied to a Mono Q column (5 × 5, Pharmacia HR 5/5) which was connected to an FPLC system (Pharmacia) and equilibrated with 20 mM Tris-HCl pH 8.0, containing 0.5 mM EGTA, 0.5 mM EDTA, 20 mM 2-mercaptoethanol, 20 μg/ml leupeptin, 50 μM *p*-APMSF, 10% (v/v) glycerol, and 0.02% (v/v) Triton X-100 (Buffer A). After washing with 10 ml of Buffer A, PKC was eluted by application of a 50-ml linear concentration gradient of NaCl (0–0.5 M) in Buffer A at a flow rate of 0.5 ml/min. Fractions of one ml each were collected, and an aliquot of each fraction was assayed for PKC activity. A single major peak of PKC fractions (20 ml, fractions 21–45) was applied directly to a hydroxyapatite column (0.78 × 15, KB-0515 type C), which was connected to the FPLC system. The hydroxyapatite column was previously equilibrated with 20 mM potassium phosphate, pH 7.5, containing 0.5 mM EGTA, 0.5 mM EDTA, 20 mM 2-mercaptoethanol, 20 μg/ml leupeptin, 50 μM *p*-APMSF, 10% (v/v) glycerol, and 0.02% (v/v) Triton X-100 (Buffer B). After washing with 20 ml of Buffer B, PKC

was eluted by application of an 84-ml linear concentration gradient of potassium phosphate (20–215 mM) in Buffer B at a flow rate of 0.4 ml/min. Fractions of one ml each were collected and assayed for enzyme activity.

Rat brain cytosolic PKC was resolved into three major peaks by hydroxyapatite column chromatography as described [12].

2.3. *Standard of PKC subspecies and assay*

The α-, β-, γ-, δ-, and ε-subspecies were purified from the rat brain as described [11–14]. The ζ-subspecies was partially purified from Chinese hamster ovary (CHO) cells over-expressing the recombinant enzyme (Watanabe et al., in preparation). The PKC subspecies were assayed by measuring the incorporation of $^{32}P_i$ into MBP from [γ-$^{32}$P]ATP. The standard reaction mixture (50 μl) contained 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 10 μM [γ-$^{32}$P]ATP, MBP (100 μg/ml), PtdSer (16 μg/ml), diolein (1.6 μg/ml) and 10 μM $CaCl_2$. After incubation for 30 min at 30°C, the reaction was terminated by spotting a 40-μl aliquot of the reaction mixture onto P81 paper (Whatman). The paper was washed five times, each time for 5 min by immersion in 10 ml of 75 mM $H_3PO_4$. The radioactivity of the paper was quantitated using a scintillation spectrometer by Cherenkov counting.

2.4. *Immunoblot analysis*

The fractions from hydroxyapatite column chromatography were subjected to SDS-PAGE, followed by electrotransfer onto Immobilon (Millipore) membranes for Western blotting. The membranes were reacted with subspecies-specific antibodies, and stained by the avidin-biotin peroxidase complex method. The antibodies against PKC subspecies designated CKpV5α-a, CKpV1β-a, CKpV3γ-a, CKpV3δ-a, CKpV5ε-a, and CKpV5ζ-a were employed [13–16]. The crude lysates from CHO cells over-expressing the recombinant δ-, ε-, and ζ-subspecies were employed as authentic enzyme samples.

## 3. RESULTS AND DISCUSSION

### 3.1. *Specificity of antibodies*

To confirm the specificity of the subspecies-specific antibodies employed, immunoblot analysis of authentic PKC subspecies was carried out (Fig. 1). The antibodies, CKpV5α-a, CKpV1β-a, CKpV3γ-a, CKpV3δ-a, CKpV5ε-a, and CKpV5ζ-a could specifically recognize αPKC (81 kDa), βPKC (80 kDa), γPKC (81 kDa), δPKC (doublet of 76 and 78 kDa; see [13]), εPKC (doublet of 90 and 93 kDa; see [14]), and ζPKC (76 kDa), respectively. No cross-reaction was observed. CKpV5ζ-a frequently reacted with a small protein of 48 kDa, which is probably a proteolytic fragment of ζPKC produced during enzyme preparation (Fig. 1F).

### 3.2. *Partial resolution of PKC subspecies*

PKC from the rat retina was resolved into several major and minor fractions by hydroxyapatite column chromatography (Fig. 2A), eluting at approx. 75 mM (peak I), at 94 mM (peak II), at 137 mM (peak III), and at 170 mM (peak IV) potassium phosphate concentration. The peak I enzyme was very small, but exactly coincided with the elution position of the γ-subspecies from brain tissues (Fig. 2B). The small peak I enzyme showed typical characteristics of PKC that required $Ca^{2+}$, diacylglycerol, and PtdSer (Fig. 2A). Although other enzyme peaks required $Ca^{2+}$ for their full activation, part of the peak II enzyme (fractions 32–40) was apparently independent of $Ca^{2+}$. Indeed, the peak II enzyme contained several PKC subspecies, which were independent of $Ca^{2+}$ for enzymatic activity (see below). The $Ca^{2+}$-dependent PKC subspecies from the rat brain are normally resolved into three distinct fractions (peak I, II and III) by hydroxyapatite column chromatography, which are corresponding to the γ-, β- (mixture of βI and βII), and α-subspecies, respectively (Fig. 2B). The δ- and ε-subspecies from the rat brain, both of which are $Ca^{2+}$-independent, have been shown to be eluted in fractions 32–40, just after the β-subspecies [13,14].

### 3.3. *Identification of PKC subspecies*

To identify the PKC subspecies expressed in rat ret-

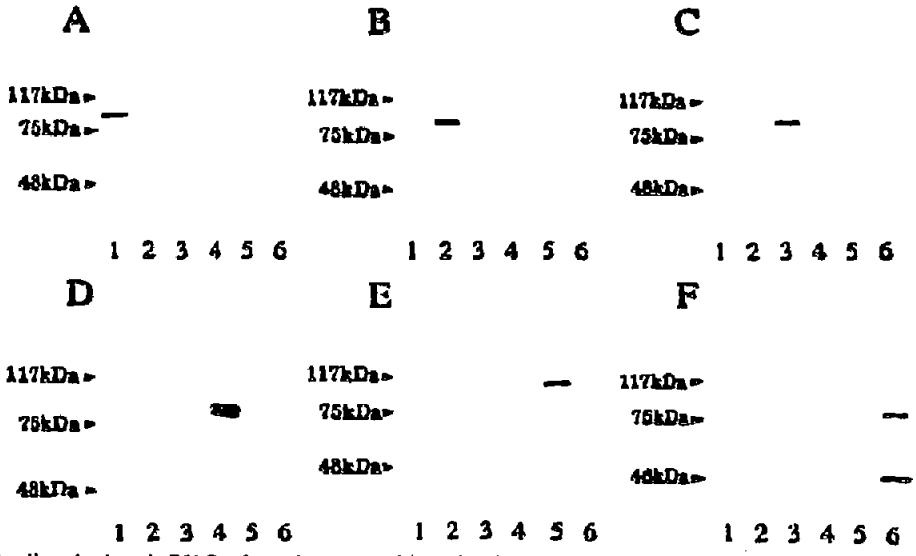


Fig. 1. Specificity of antibodies. Authentic PKC subspecies were subjected to immunoblot analysis. (A–F). Immunoblot with the subspecies-specific antibodies, CKpV5α-a, CKpV1β-a, CKpV3γ-a, CKpV3δ-a, CKpV5ε-a, and CKpV5ζ-a, respectively. Lane 1, αPKC; lane 2, βPKC; lane 3, γPKC; lane 4, δPKC; lane 5, εPKC; and lane 6, ζPKC.

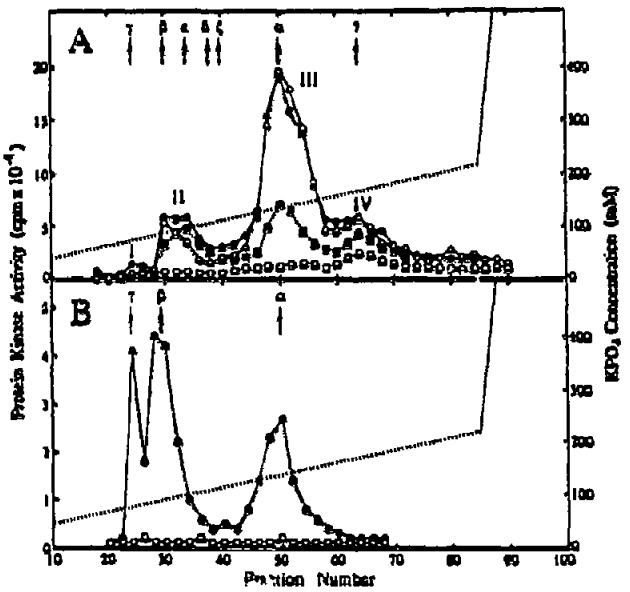


Fig. 2. Partial resolution of PKC subspecies in rat retina by hydroxyapatite column chromatography. (A) PKC subspecies from rat retina; (B) PKC subspecies from rat brain cytosol. PKC activity was assayed with MBP as substrate under the conditions described in section 2. (●), assayed in the presence of PtdSer (16 μg/ml), diolein (1.6 μg/ml), and $CaCl_2$ (10 μM); (○), assayed in the presence of PtdSer and $CaCl_2$; (■), assayed in the presence of PtdSer, diolein, and EGTA (0.5 mM); and (□), assayed in the presence of EGTA alone. (·····) potassium phosphate concentration.

ina. immunoblot analysis of the hydroxyapatite column fractions described above was made with subspecies-specific antibodies (Fig. 3). The results showed that peak II contained not only the β-subspecies (fractions 30–34) but also the ε- (fractions 30–36), δ- (fractions 34–42), and ζ-subspecies (fractions 34–52). Peak III contains mainly the α-subspecies (fractions 46–68) and a little quantity of the ζ-subspecies. Peak I corresponded to the γ-subspecies, indicating that the retina expresses a very little, if any, of this subspecies. This amount of the peak I enzyme was not sufficient enough for the immunoblot detection. Recent reports from several laboratories [9,10] describe conflicting results for the exis- tence of the γ-subspecies in retinal tissue. This conflict is probably due to an extremely small amount of this PKC subspecies in this tissue, and also the result of the detection limit by the immunological procedures employed. It is possible that this subspecies is only expressed in specific neuronal cells with limited intracellular localization in the retina.

Peak IV appeared to be contaminated by the α-subspecies, but contained a structurally unknown member of the PKC family.

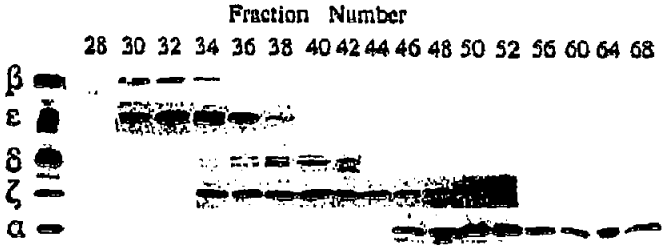


Fig. 3. Immunoblot analysis of PKC subspecies from rat retina. Each fraction eluted from hydroxyapatite column was subjected to immunoblot analysis using the subspecies-specific antibodies, $CKpV_1\beta$-a for βPKC, $CKpV_1\varepsilon$-a for εPKC, $CKpV_1\delta$-a for δPKC, $CKpV_1\zeta$-a for ζPKC, and $CKpV_1\alpha$-a for αPKC, respectively. The detailed procedures are described in section 2.

### 3.4. *Kinetic properties of PKC subspecies*

The PKC family has been conventionally divided into two subgroups, $Ca^{2+}$-dependent (α, β and γ) and $Ca^{2+}$-independent (δ, ε, ζ and η) enzymes [1–3]. Some of the kinetics of these subspecies are given in Fig. 4A–E. On hydroxyapatite column chromatography the β-, δ-, ε-, and ζ-subspecies were overlapping and not sharply resolved. For example, enzyme fraction 30 predominantly contained the β-subspecies together with a small amount of the ε-subspecies. Thus, this fraction showed properties resembling the β-subspecies (Fig. 4F). The enzyme fraction 34 predominantly contained the ε-subspecies and small quantities of the δ-, ζ- and β-subspecies. Thus, this fraction showed properties of a mixture of the $Ca^{2+}$-dependent and independent enzymes (Fig. 4G). Thus far we have not been able to sharply separate the β-, δ-, ε- and ζ-subspecies free of one another by chromatography on a hydroxyapatite column. The enzyme in peak IV has not been identified. Although the existence of the η-subspecies has not yet been identified in this tissue, the results briefly described above indicate that the retinal tissue expresses most of the PKC subspecies so far found in mammals. The precise cellular and intracellular localization of these subspecies will be clarified by subsequent studies.

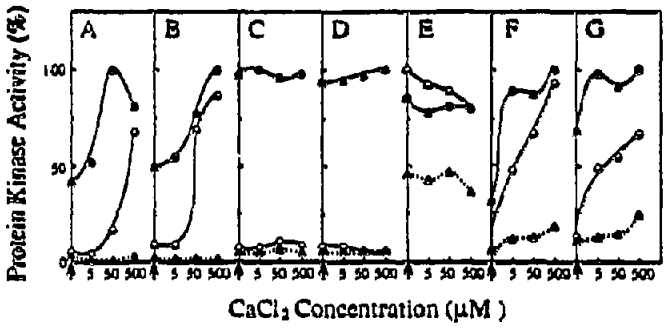


Fig. 4. Kinetic properties of PKC subspecies. PKC activity was assayed at various concentrations of $CaCl_2$. (A–E) Authentic enzyme samples of αPKC, βPKC, δPKC, εPKC, and ζPKC, respectively. (F,G) Rat retina PKC (fraction 30) and rat retina PKC (fraction 34), respectively. Where indicated with an arrow, 0.5 mM EGTA was added instead of $CaCl_2$. (●), assayed in the presence of PtdSer (16 μg/ml) and diolein (1.6 μg/ml); (○), assayed in the presence of PtdSer alone; and (▲), assayed in the absence of PtdSer and diolein.

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