

## Activation of a brain-specific protein kinase C subspecies in the presence of phosphatidylethanol

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Protein kinase C (PKC) is normally activated by diacylglycerol in the presence of  $\text{Ca}^{2+}$  and phosphatidylserine. At physiological concentrations of  $\text{Ca}^{2+}$ , however, phosphatidylethanol, a product of the phospholipase D-catalyzed transphosphatidyl reaction between membrane phospholipids and ethanol, can replace phosphatidylserine, and activate PKC. This mode of activation is most effective for the  $\gamma$ -subspecies, which is expressed only in central nervous tissue. Phosphatidylmethanol is also effective to some extent. Consideration of these results suggests the possibility that ethanol may exert some effect on signal transduction in this tissue via changes in protein phosphorylation.

Protein kinase C; Phosphatidylethanol; Ethanol; Phospholipase D

### 1. INTRODUCTION

Protein kinase C (PKC) is normally activated by diacylglycerol in the presence of  $\text{Ca}^{2+}$  and acidic phospholipids (review [1]). At physiologically low concentrations of  $\text{Ca}^{2+}$ , phosphatidylserine is indispensable for the enzyme activation [2]. Phospholipase D has been known for some time to catalyze the transphosphatidyl reaction between various membrane phospholipids and alcohols such as ethanol [3,4]. In this reaction phosphatidylcholine is the most effective phosphatidyl donor [3]. In fact, phosphatidylethanol has been found in the brain, heart, kidney, liver

and skeletal muscle tissue of ethanol-treated rats [5–7].

Recent analysis of cDNA clones indicates the presence of several subspecies of PKC in brain tissues (review [8]). The brain PKC can be resolved into three types, type I, II and III, upon chromatography on a hydroxyapatite column [9–11]. The structure of each type has been identified by comparison with the enzymes that were separately expressed in COS 7 cells transfected by the respective cDNA-containing plasmids [10,11]. Type I has the structure encoded by  $\gamma$ -sequence; type II is a mixture of the subspecies having  $\beta$ I- and  $\beta$ II-sequences, which are derived from a single RNA transcript by alternative splicing; and type III has the structure of  $\alpha$ -sequence. These three types show subtle individual characteristics with slightly different kinetic and catalytic properties [12,13]. Immunocytochemical and biochemical analysis has shown that the type I enzyme is expressed only in central nervous tissues [14,15]. This communication will describe that, among various subspecies tested, this brain-specific PKC is particularly sensitive to phosphatidylethanol for its activation.

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The data are taken in part from the dissertation that will be submitted by Y. Asaoka to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Science. Part of the data contained in this paper was reported in a preliminary form at the 625th meeting (London) of the Biochemical Society.

## 2. MATERIALS AND METHODS

### 2.1. Materials and chemicals

Phosphatidylethanol and phosphatidylmethanol were prepared as described [16]. Phosphatidylserine and 1,2-diolein were obtained from Serdary Research Laboratories. Phosphatidylethanolamine was obtained from Avanti Polar-Lipids.

### 2.2. Purification and assay of PKC

PKC was purified from the rat brain soluble fraction by DE-52, threonine-Sepharose and TSK phenyl-5PW column chromatographies, and was separated into three types, type I, II and III, by chromatography on a hydroxyapatite column connected to a Pharmacia FPLC system as described [10,11]. Each type of PKC was apparently homogeneous upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis. PKC was assayed as described [17]. The reaction mixture (0.25 ml) contained 20 mM Tris-HCl at pH 7.5, 200  $\mu$ g/ml calf thymus H1 histone, 10  $\mu$ M [ $\gamma$ - $^{32}$ P]ATP (50–150 cpm/pmol), 5 mM magnesium acetate, 0.01 mM EGTA (from enzyme fraction), each type of PKC (approximately 0.05  $\mu$ g), and various amounts of  $\text{CaCl}_2$ , phospholipids and 1,2-diolein, as indicated in each experiment. Phospholipids and 1,2-diolein were mixed in a small amount of chloroform, dried under a nitrogen stream, and dispersed in 20 mM Tris-HCl at pH 7.5 by vigorous vortex mixing followed by sonication for 5 min at 4°C. The incubation was carried out for 3 min at 30°C, and the reaction was terminated by the addition of 25% of trichloroacetic acid. Acid precipitable materials were collected on a nitrocellulose membrane and the radioactivity was quantitated by liquid scintillation counter.

Table 1

Distribution of PKC activity between the enzyme subspecies isolated from the soluble fraction of various tissue preparations

| Tissue          | Protein kinase activity (% total) |         |          |
|-----------------|-----------------------------------|---------|----------|
|                 | Type I                            | Type II | Type III |
| Whole brain     | 26.1                              | 49.1    | 24.8     |
| Cerebral cortex | 19.8                              | 62.7    | 17.5     |
| Hippocampus     | 24.9                              | 40.5    | 34.6     |
| Cerebellum      | 52.1                              | 33.5    | 14.4     |
| Spinal cord     | 3.2                               | 49.7    | 47.1     |
| Sciatic nerve   | –                                 | 7.1     | 92.9     |
| Liver           | –                                 | 31.3    | 68.7     |
| Spleen          | –                                 | 67.5    | 32.5     |
| Kidney          | –                                 | 18.3    | 81.7     |

Tissue samples were homogenized in 20 mM Tris-HCl at pH 7.5 containing 0.25 M sucrose, 10 mM EGTA, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 20  $\mu$ g/ml leupeptin, and centrifuged at  $100\,000 \times g$  for 60 min. The supernatant was applied to a DEAE-cellulose column, followed by resolution of the partially purified enzyme subspecies on a hydroxyapatite column to a FPLC system (Pharmacia). PKC activity was assayed with 0.3 mM  $\text{CaCl}_2$ , 0.8  $\mu$ g/ml 1,2-diolein and 8  $\mu$ g/ml phosphatidylserine as described in section 2

## 3. RESULTS

As described elsewhere [9–11], the brain PKC was resolved into three major fractions, types I, II and III, upon hydroxyapatite column chromatography. Table 1 shows the relative distribution of the enzyme activity between the enzyme subspecies present in several regions of the brain and some other tissues. The identity of each type was confirmed by immunoblot analysis with type-specific antibodies. The type II enzyme fraction is an unequal mixture of  $\beta$ I- and  $\beta$ II-subspecies which can be distinguished thus far only by immunological procedures. The results show that a differential pattern of expression of the various subspecies is apparent in the tissues examined. In particular, type I enzyme encoded by  $\gamma$ -sequence was found in the brain and spinal cord, but not in any of the other tissues or cell types so far tested.

The enzyme subspecies isolated from various tissues required phospholipid for their catalytic activity, and phosphatidylserine was the most active at physiologically low concentrations of  $\text{Ca}^{2+}$ . Table 2 shows the specificity of various phospholipids to support the enzymatic activity of the three types of PKC from rat whole brain. Phosphatidylinositol could activate the enzymes, and in addition both phosphatidylethanol and phosphatidylmethanol could substitute for phosphatidylserine,

Table 2

Activation of types I, II and III PKC by various phospholipids

| Phospholipid added                      | Protein kinase activity (%) |         |          |
|-----------------------------------------|-----------------------------|---------|----------|
|                                         | Type I                      | Type II | Type III |
| Phosphatidylserine (8 $\mu$ g/ml)       | 100                         | 100     | 100      |
| Phosphatidylinositol (8 $\mu$ g/ml)     | 65                          | 32      | 60       |
| Phosphatidylethanolamine (8 $\mu$ g/ml) | 16                          | 15      | 9        |
| Phosphatidylcholine (8 $\mu$ g/ml)      | 3                           | 2       | 2        |
| Sphingomyelin (5 $\mu$ g/ml)            | 8                           | 3       | 2        |
| Phosphatidylethanol (5 $\mu$ g/ml)      | 67                          | 31      | 28       |
| Phosphatidylethanol (20 $\mu$ g/ml)     | 63                          | 24      | 23       |
| Phosphatidylmethanol (5 $\mu$ g/ml)     | 48                          | 45      | 44       |
| Phosphatidylmethanol (20 $\mu$ g/ml)    | 46                          | 31      | 31       |
| None                                    | 7                           | 7       | 7        |

PKC was assayed with 3  $\mu$ M  $\text{CaCl}_2$ , 0.8  $\mu$ g/ml 1,2-diolein and each phospholipid as indicated. Results are normalized to the activity obtained with phosphatidylserine. Other conditions are described in section 2

particularly for the activation of type I PKC. It was confirmed that at relatively higher concentrations of  $\text{Ca}^{2+}$  (0.3 mM) acidic phospholipids but not basic phospholipids could activate the enzyme. Fig.1 provides further evidence that for the activation of type I enzyme phosphatidylserine can be replaced by phosphatidylethanol and to a lesser extent by phosphatidylmethanol (fig.1A). These phosphatidylalcohols were far less effective for type II and type II enzyme (fig.1B and 1C). Neither ethanol nor methanol affected the enzyme activation at comparable concentrations. The result given in table 3 shows that the activation of type I enzyme by phosphatidylalcohol is dependent of diacylglycerol.

#### 4. DISCUSSION

Phospholipase D of plant origin was reported as early as 1967 by Dawson [18] and Yang et al. [16] to catalyze the phosphatidyltransferase reaction. Mammalian phospholipase D has been subsequently shown to produce phosphatidylethanol [3,4], and the brain synaptosomal membrane possesses the highest activity [3]. It is worth noting that the phosphatidylethanol formation requires unsaturated fatty acids such as arachidonate and oleate, and that phosphatidylcholine is the most ef-

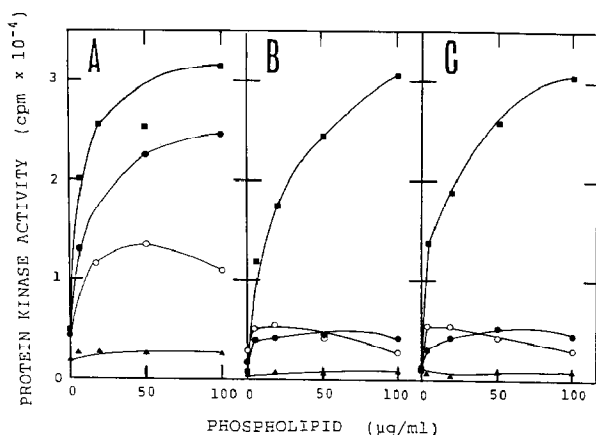


Fig.1. The activation of three types of PKC by various concentrations of phospholipids. PKC was assayed with 3  $\mu\text{M}$   $\text{CaCl}_2$ , 0.8  $\mu\text{g/ml}$  1,2-diolein, and various concentrations of each phospholipid as described in section 2. (A) Type I; (B) type II; (C) type III. (●) Phosphatidylethanol; (○) phosphatidylmethanol; (■) phosphatidylserine; (▲) phosphatidylethanolamine.

Table 3

Effect of 1,2-diolein on type I PKC

| Phospholipid and diacylglycerol added |               | Protein kinase activity |     |
|---------------------------------------|---------------|-------------------------|-----|
|                                       |               | cpm                     | %   |
| Phosphatidylserine                    | + 1,2-diolein | 13 880                  | 100 |
|                                       | - 1,2-diolein | 2 480                   | 8   |
| Phosphatidylethanol                   | + 1,2-diolein | 11 190                  | 78  |
|                                       | - 1,2-diolein | 3 760                   | 19  |
| Phosphatidylmethanol                  | + 1,2-diolein | 13 400                  | 96  |
|                                       | - 1,2-diolein | 3 380                   | 16  |
| none                                  |               | 1 450                   | -   |

Type I PKC was assayed with 3  $\mu\text{M}$   $\text{CaCl}_2$ , 4  $\mu\text{g/ml}$  of each phospholipid, and with or without 0.8  $\mu\text{g/ml}$  1,2-diolein, as described in section 2

fective phosphatidyl donor [3]. Although ethanol is one of the most common psychotropic agents, the mechanism of its behavioral and neurological effects remains to be clarified. At present, it is generally thought that ethanol exerts its biological effects on the lipids of the cell membranes [19]. It was only recently that the presence of phosphatidylethanol was in fact demonstrated in various tissues from ethanol-treated rats [5-7]. The present studies suggest that this species of phospholipid can substitute for phosphatidylserine to activate PKC. This activation is most potent for the type I enzyme, encoded by  $\gamma$ -sequence, which is apparently present only in the brain and spinal cord. This enzyme subspecies is abundant in the cerebellar cortex (Purkinje cells), hippocampus (pyramidal cells) and cerebral cortex. An earlier report [2] has shown that, although phosphatidylserine is indispensable, acidic phospholipids can potentiate the activation of PKC whereas phosphatidylcholine and sphingomyelin are inhibitory. A possibility open to further study, therefore, is that ethanol may exert some of its biological actions on neuronal tissues by modulating signal transduction through changes in protein phosphorylation.

#### NOTE ADDED IN PROOF

We have recently learned that Dr Mordechai Liscovitch of the Department of Hormone Research, the Weizmann Institute, observed a stimulatory effect of phosphatidylethanol on an

stimulatory effect of phosphatidylethanol on an unfractionated mixture of rat brain PKC preparations (personal communication).

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