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Characterization of a Zn^{2+}-requiring glycerophosphocholine cholinephosphodiesterase possessing *p*-nitrophenylphosphocholine phosphodiesterase activity

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p-Nitrophenylphosphocholine phosphodiesterase activity was purified 5000-fold from mouse brain by treatment of membranes with *Bacillus cereus* phospholipase C preparation and sequential chromatographies on concanavalin A–Sepharose and CM-Sephadex columns. The phosphodiesterase (Zn^{2+}-requiring) showed K_m and $V_{max.}$ values of 5.5 μM and 4.2 μmol/min per mg respectively in the hydrolysis of *p*-nitrophenylphosphocholine, and possessed an optimum pH of 10.5 and a molecular mass of approx. 74 kDa. The purified enzyme was found to convert glycerophosphocholine into glycerol and phosphocholine, with K_m and $V_{max.}$ of 48 μM and 5 μmol/min per mg respectively. In the hydrolysis of glycerophosphocholine the enzyme also exhibited a Zn^{2+} requirement and optimal pH at 10.5. Additionally, the *p*-nitrophenylphosphocholine phosphodiesterase activity was competitively inhibited by glycerophosphocholine, with a K_i value of 50 μM. These observations, together with chromatographic behaviour and heat-denaturation analyses, indicate that both *p*-nitrophenylphosphocholine phosphodiesterase and glycerophosphocholine cholinephosphodiesterase activities reside in the same protein.

INTRODUCTION

Although many types of phosphodiesterases had been reported to hydrolyse various chromogenic substrates containing the *p*-nitrophenyl group [1–4], there has been no report concerning the mammalian enzyme responsible for the hydrolysis of *p*-nitrophenylphosphocholine (*p*-NPPC), except the observations that sperm [5] or myelin [6] catalysed the hydrolysis of *p*-NPPC. Especially, the myelin from rat brain was reported to cleave *p*-nitrophenyl phosphodiesters such as *p*-NPPC, *p*-nitrophenylthymidine and bis(*p*-nitrophenyl)phosphate. Subsequently, the *p*-NPPC phosphodiesterase was partially purified from mouse brain, and shown to possess the optimum pH of 11 and no dependence on Ca^{2+} [7]. Separately, a particulate *p*-NPPC phosphodiesterase present in various subcellular fractions of rat brain was reported to exhibit an optimum pH of 8.5 and a requirement for Zn^{2+} or Ca^{2+} [8]. In our further studies, *p*-NPPC phosphodiesterase, which was solubilized from brain membranes by microbial phosphatidylinositol-specific phospholipase C (PI-phospholipase C), was found to be different from the detergent-solubilized enzyme in its ability to associate with membranes and thermostability (D.-E. Sok & M. R. Kim, unpublished work).

Meanwhile, a phosphodiesterase activity catalysing the conversion of glycerophosphocholine (GPC) into phosphocholine and glycerol had been reported to be present in brain tissue [9]. Later, the GPC cholinephosphodiesterase (EC 3.1.4.38), present in both soluble form (25%) and bound form (75%), was reported to possess a molecular mass of 240 kDa and a dependence on Ca^{2+} [10]. More recently [6], another type of GPC cholinephosphodiesterase which shows optimum pH 9.5 and an absolute requirement for Zn^{2+} was reported to be present in the myelin part of rat brain. Although the myelin was observed to possess the enzyme activity to hydrolyse both *p*-NPPC and GPC, and the same enzyme was suggested to be responsible for the hydrolysis of two phosphodiesters [6], the purification or characterization of the phosphodiesterase has not been attempted. Therefore, it has been intriguing to purify and characterize the *p*-NPPC phosphodiesterase. Here, we introduce a procedure of purifying the *p*-NPPC phosphodiesterase from mouse brain, and propose that the purified enzyme corresponds to one type of glycerophosphocholine cholinephosphodiesterase requiring Zn^{2+}.

MATERIALS AND METHODS

Materials

p-NPPC, GPC, concanavalin A(Con A)–Sepharose, glycerol kinase, glycerol-phosphate oxidase, horseradish peroxidase, choline kinase, glycerol, ATP, CM-Sephadex, Sephadex G-150, PI-phospholipase C (*Bacillus cereus*, Type III), EDTA, and other phosphocholine derivatives were products of Sigma Chemical Co. (St. Louis, MO, U.S.A.). The h.p.l.c. gel column and protein standards were produced by Waters Associates (Milford, MA, U.S.A.) and Bio-Rad (Richmond, CA, U.S.A.) respectively.

Enzyme assay

p-NPPC phosphodiesterase activity was determined by measurement of *p*-nitrophenol released from the hydrolysis of *p*-NPPC [4,7]. The increase in A_{415} was monitored spectrophotometrically. Unless otherwise described, assays were conducted in 1 ml of 0.1 M-glycine buffer, pH 9.5, containing 150 μM-*p*-NPPC at 20 °C. The enzyme activity (units) was expressed in μmol of *p*-nitrophenol produced/min. Protein was determined quantitatively by the method of Bradford [11]. For measurement of enzyme activity in the organs, the tissue (0.2 g) was homogenized in 2 ml of 0.1 M-glycine buffer, pH 10.5, and a 50 μl portion was incubated with 150 μM-*p*-NPPC in 2 ml of the same buffer containing 1 mM-EDTA and 0.2% Triton 100. Separately, GPC cholinephosphodiesterase activity was measured by an enzymic

Abbreviations used: *p*-NPPC, *p*-nitrophenylphosphocholine; GPC, glycerophosphocholine; Con A, concanavalin A; PI-phospholipase C, phosphatidylinositol-specific phospholipase C.

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method employing glycerol kinase and glycerol-phosphate oxidase [12,13]. The enzyme was incubated with 1.25 mM-GPC in 1 ml of 0.1 M-glycine buffer, pH 10.5, for 1 h at 20 °C. Then the above mixture was adjusted to pH 7.0, and mixed with 1 ml of 0.1 M-phosphate buffer, pH 7.0, including glycerol kinase (1 unit), glycerol-phosphate oxidase (2 units), horseradish peroxidase (2 units), ATP (0.3 μmol), $MgCl_2$ (3 μmol) 4-aminoantipyrine (3 μmol) and sodium *N*-ethyl-*N*-(3-sulphopropyl)-*m*-anisidine, and the increase in A_{540} was measured. The glycerol produced was determined by using the calibration curve with standard glycerol (0–240 nmol/ml). Separately, choline was measured by the modified method of the enzyme-coupled assay [14–16]: the enzyme was incubated with 1.25 mM substrate in 1 ml of 0.1 M-glycine buffer, pH 9.5, containing 20 units of alkaline phosphatase, and to the mixture were added choline oxidase (2 units), peroxidase (50 units) and 1 mM-azobis-(3-ethyl-2,3-dihydroxybenzothiazoline-6-sulphonate).

Preparation of soluble *p*-NPPC phosphodiesterase

Mouse brains (50 heads) were homogenized in 10 vol. of 10 mM-Tris buffer, pH 7.4, containing 0.25 M-sucrose and centrifuged at 50000 *g* for 20 min. The precipitate was re-washed with the above buffer and re-centrifuged, and the pellet was diluted with 10 vol. of 0.1 M-Tris buffer, pH 7.0. After the 10% homogenate (100 ml) was incubated with 100 units of phospholipase C at 37 °C for 75 min, the mixture was centrifuged (50000 *g*, 20 min) at 4 °C, and the supernatant was used as the enzyme extract (80 ml).

Partial purification

The enzyme extract (80 ml) was applied to a Con A–Sepharose column (1.9 × 7 cm), which was washed with 10 mM-Tris buffer, pH 7.4, containing 0.5 M-NaCl, and the bound enzyme activity was eluted with 0.25 M-α-methyl mannoside as described in Legends. The fractions expressing phosphodiesterase activity were pooled, dialysed against 10 mM-Tris buffer, pH 7.0, and loaded on a CM-Sephadex (0.9 cm × 15 cm) column, which was eluted at the rate of 1.4 ml/min with 10 mM-Tris buffer, pH 7.4, containing a concentration gradient of NaCl. The portions (58–64 min) possessing the *p*-NPPC phosphodiesterase activity were pooled, concentrated and applied to a Sephadex G-150 column (1.9 cm × 60 cm), which was eluted at 24 ml/h with 10 mM-Tris buffer, pH 7.4, containing 0.1 M-NaCl.

The molecular mass of the protein was determined by h.p.l.c. using a Protein PAK 300 SW h.p.l.c. column (0.8 cm × 30 cm), which was eluted at 1 ml/min with 0.1 M-phosphate buffer (pH 7) containing 0.5 M-NaCl, and SDS/PAGE was carried out as described by Laemmli [17].

Enzymic analyses

The kinetic parameters were obtained from Lineweaver–Burk plots, and general properties of the phosphodiesterase were obtained as described in Figure legends. To determine the effect of pH on the enzyme, the activity was measured at various pH values (pH 8–11). To study the metal requirement, the purified enzyme was dialysed against a solution of 0.32 M-sucrose and 1 mM-EDTA overnight, and the sample was then dialysed overnight in the cold against 0.32 M-sucrose to remove EDTA. Several bivalent cations were added to the doubly dialysed sample, and the restored enzyme activity was expressed as the percentage of original activity. Separately, the thermostability of the enzyme activity was determined by measuring the remaining activity after incubation of the purified enzyme with the respective substrate in 0.1 M-glycine buffer, pH 9.5, at various temperatures (36–52 °C) for 5 min. Additionally, the phosphodiesterase activity of each fraction from Sephadex G-150 gel chromatography was assayed with both GPC and *p*-NPPC as substrates as described above.

RESULTS

Since brain tissue with a specific activity of 17 ± 4.6 nmol/h per mg tissue weight had a higher *p*-NPPC phosphodiesterase activity than kidney (1.9 ± 1.0 nmol/h per mg) or liver tissue (< 0.8), the enzyme purification was attempted from brain homogenate, where more than 90% of the enzyme activity was found to be tightly bound to membranes [7]. Incubation of the brain membrane with *B. cereus* PI-phospholipase C preparation resulted in liberation of over 90% of membrane-bound *p*-NPPC-hydrolysing activity. The released phosphodiesterase activity was partially purified by Con A–Sepharose chromatography (Fig. 1). Most of the total activity bound to the Con A column, and the bound enzyme activity was eluted selectively by α-methyl manno-

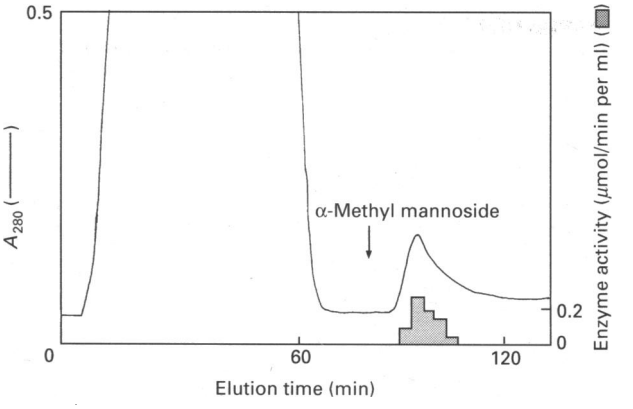

Fig. 1. Con A–Sepharose chromatography

The enzyme extract (80 ml) was applied to a column of Con A–Sepharose (1.9 cm × 7 cm), which was washed with 10 mM-Tris buffer, pH 7.4, containing 0.5 M-NaCl. The enzyme was eluted with the same buffer containing 0.25 M α-methyl mannoside (flow rate 24 ml/h).

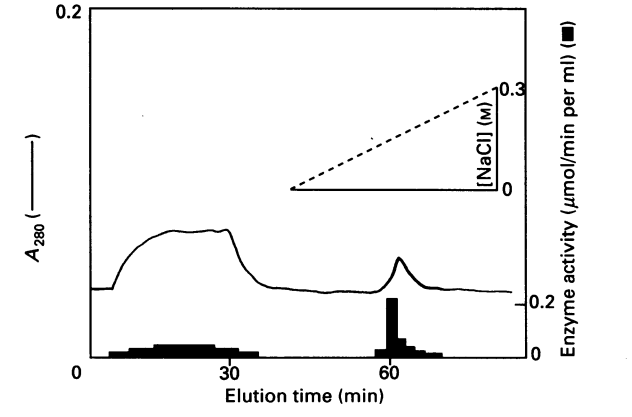

Fig. 2. CM-Sephadex chromatography

The enzyme sample from Con-A column was applied to a CM–Sephadex column (0.9 cm × 15 cm), which was equilibrated with 10 mM-Tris buffer, pH 7.4. The enzyme was eluted with the same buffer containing a concentration gradient of NaCl (----) (flow rate 1.4 ml/min).

Table 1. Purification of *p*-NPPC phosphodiesterase from mouse brain membranes

Step	Total activity (μmol/h)	Specific activity (μmol/h per mg)	Recovery (%)	Purification (fold)
Membrane homogenate	75	0.055	100	1
PI-phospholipase C supernatant	68	0.6	91	11
Con A–Sepharose eluate	54	30	72	550
CM-Sephadex eluate	27	270	36	4990
Sephadex G150 eluate	7.2	250	9.6	4550

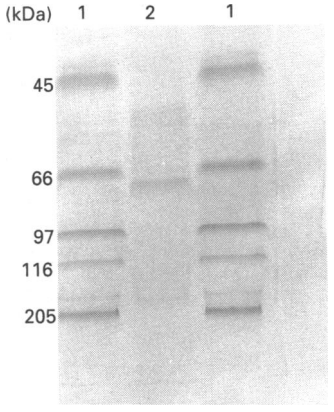

Fig. 3. SDS/PAGE of purified phosphodiesterase

The protein sample was analysed by slab-gel electrophoresis on SDS/polyacrylamide after incubation in 0.06 M-Tris buffer, pH 7.0, containing 1% SDS. Staining was performed with Coomassie Blue. Lane 1, protein standards (rabbit muscle myosin, 205 kDa; *Escherichia coli* β-galactosidase, 116 kDa; rabbit muscle phosphorylase *b*, 97 kDa; BSA, 66 kDa; ovalbumin, 45 kDa). Lane 2, purified *p*-NPPC phosphodiesterase.

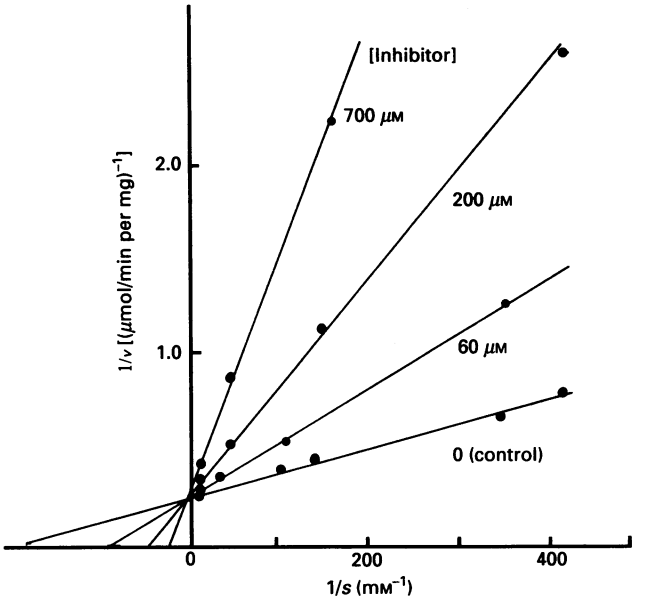

Fig. 4. Inhibitory effect of GPC on the rate of *p*-NPPC hydrolysis

The purified enzyme (2 m-units) was incubated with various concentrations of *p*-NPPC in the absence or presence of GPC as an inhibitor, and the rate of hydrolysis was measured, as described in the Materials and methods section.

Table 2. Effects of metal ions on the activity of the phosphodiesterase dialysed against 1 mM-EDTA

The twice-dialysed enzyme was preincubated with the metal ion for 30 min at 20 °C. The restored enzyme activity was determined with *p*-NPPC and GPC as substrates as described in the Materials and methods section, and values are expressed as averages of two or three measurements.

Bivalent ion	Concn. (mM)	Restored activity (%)	
		p-NPPC	GPC
$CaCl_2$	1	0	0
$MgCl_2$	1	0	0
$MnSO_4$	1	0	0
$ZnCl_2$	0.01	18	19
	0.03	39	37
	0.1	58	54
	1	89	90

side, with a specific activity of 0.5 μmol/min per mg and a recovery of 90%. From these results, it is suggested that the phosphodiesterase is one kind of glycosylphosphatidylinositol-anchored glycoprotein [18]. Subsequently, the fractions (95–110 min) from Con A–Sepharose chromatography were pooled, dialysed, and applied to a CM-Sephadex column. As demonstrated in Fig. 2, approximately two-thirds of the total phosphodiesterase activity was associated with the CM-Sephadex column, although the remaining portion appeared in the flow-through fractions. The enzyme activity bound to the column was eluted at approx. 100 mM-NaCl (Fig. 2), suggesting that the activity (60–70%) associable with the column is cationic at neutral pH. In the following step, using Sephadex G-150 gel chromatography, it was shown that the *p*-NPPC phosphodiesterase activity appeared in the fractions corresponding to a molecular mass of between 67 and 94 kDa.

Although the *p*-NPPC phosphodiesterase activity was separated with high purification (fold) by Con A–Sepharose and CM-Sephadex chromatographies, Sephadex G-150 gel-permeation chromatography did not lead to further purification. This may be due to the substantial loss of enzyme activity during this gel chromatography. As the results (Table 1) of a typical purification demonstrate, the phosphodiesterase was purified approx. 5000-fold, and obtained in 10% yield, starting from the homogenate. When the purified enzyme was subjected to molecular-mass determination by gel-permeation h.p.l.c. analysis in high-salt buffer, a value of approx. 75 kDa was found. In the SDS/PAGE analysis (Fig. 3), it seemed that the enzyme appeared as a relatively pure protein with a molecular mass of 74 kDa, although a trace of contaminants was present. In an attempt to ascertain the phosphodiesterase-catalysed formation of *p*-nitrophenol from *p*-NPPC, the purified enzyme was incubated with *p*-NPPC in the presence of alkaline phosphatase or propranolol, an inhibitor of

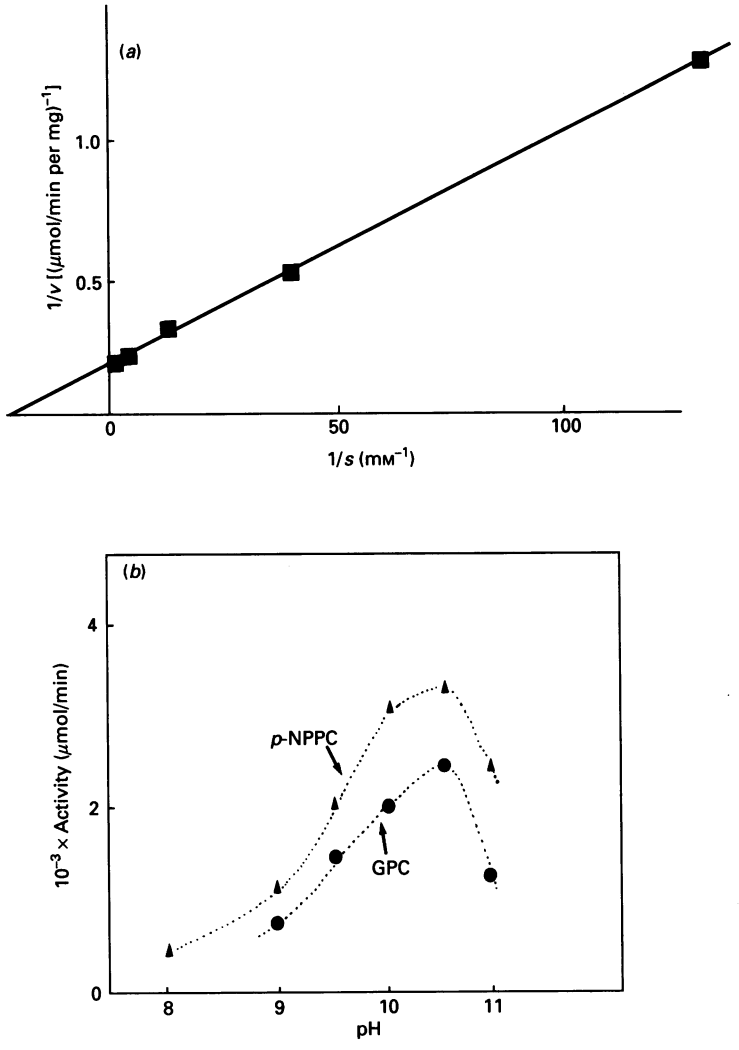

Fig. 5. (*a*) Effect of GPC concentration and (*b*) effect of pH on the rate of enzymic hydrolysis of GPC

(*a*) The enzyme (2 m-units) was incubated with various concentrations of GPC in 1 ml of 0.1 M-glycine buffer, pH 10.5, for 1 h, and the product was measured as described in the Materials and methods section. (*b*) The enzyme (2 m-units) was incubated with 1.25 mM-GPC in 1 ml of the buffer (0.1 M) of the respective pH for 1 h. For comparison, the enzyme was incubated with 150 μM-*p*-NPPC as described above.

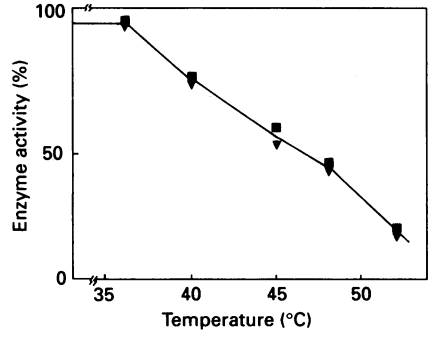

Fig. 6. Heat treatment of phosphodiesterase activities

The enzyme (2 m-units) was preincubated for 5 min in 0.1 M-glycine buffer, pH 9.5, at various temperatures. After cooling the enzyme solution to 4 °C, the phosphodiesterase activity was measured with both *p*-NPPC (■) and GPC (▼).

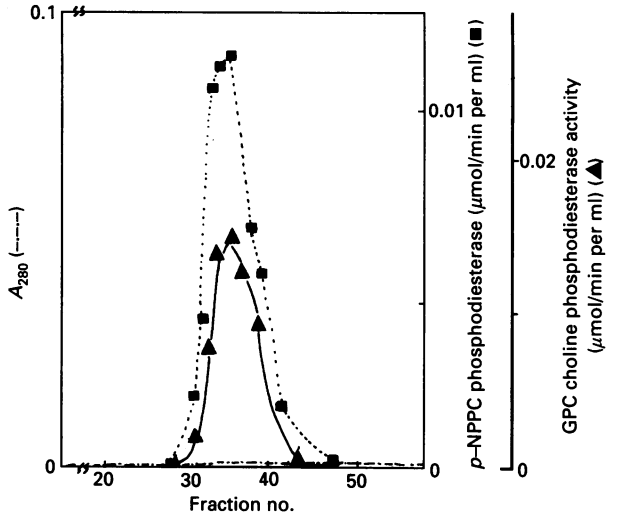

Fig. 7. Chromatography of phosphodiesterase activities on Sephadex G-150

The fractions (2 ml) from Sephadex gel chromatography were assayed for both *p*-NPPC phosphodiesterase and GPC choline-phosphodiesterase activities as described in the Materials and methods section.

phosphatase. Separately, the possible formation of choline from *p*-NPPC was investigated by enzymic measurement of choline [15,16]. It was found that the formation of *p*-nitrophenol was not affected by phosphatase or propranolol, and no formation of choline from *p*-NPPC was observed. These results confirm the above assumption that the conversion of *p*-NPPC into *p*-nitrophenol is ascribed to the direct action of the purified phosphodiesterase. The possibility that the purified enzyme preparation is contaminated with any phospholipase C activity is excluded by the additional result that the enzyme preparation did not hydrolyse phosphatidylcholine (D.-E. Sok & M. K. Kim, unpublished work).

In kinetic studies, it is shown that the formation of *p*-nitrophenol from *p*-NPPC is a linear function of both the reaction time and the amount of the purified enzyme, as reported previously [7]. When the enzyme activity was measured as a function of *p*-NPPC concentration, the kinetic properties of the enzyme followed the Michaelis–Menten equation, with a K_m of 5.5 μM and a $V_{max.}$ of 4.2 μmol/min per mg. Although the purified enzyme was found to possess an alkaline pH optimum of around 10.5, as observed previously [7], the enzyme was more stable at neutral pH than at alkaline pH. Although the simultaneous incubation with EDTA had no significant effect during a short incubation time (5 min), as reported previously [7], prior incubation of the enzyme with EDTA led to a complete loss of the enzyme activity. In the subsequent experiment (Table 2) where the metal ion requirement was investigated, it was found that the enzyme exhibits an absolute requirement for Zn^{2+} for activity, and other ions were without effect. Among the phosphocholine-group-containing compounds, only GPC exerted an inhibitory effect, although lysophosphatidylcholine showed a slightly activating effect [7]. When the inhibitory effect of GPC was investigated and analysed according to a Lineweaver–Burk plot (Fig. 4), GPC was found to exhibit the inhibition pattern as a typical competitive inhibitor with K_i of approx. 50 μM. These results led to the assumption that GPC might be an intrinsic substrate for the purified phosphodiesterase.

In the following experiment, where the enzymic-hydrolysis product of GPC was analysed, it was observed that the enzyme

preparation converted GPC into glycerol and phosphocholine with a specific activity of 5 μmol/min per mg and a K_m of 48 μM (Fig. 5*a*), and the optimum pH for hydrolysis of GPC was close to that observed with the hydrolysis of *p*-NPPC (Fig. 5*b*). From these observations, it was assumed that the *p*-NPPC phosphodiesterase is a GPC cholinephosphodiesterase. To study the identity of both *p*-NPPC phosphodiesterase activity and GPC cholinephosphodiesterase activity, comparison experiments were carried out. When the thermal denaturation of the enzyme was investigated with two types of substrates (Fig. 6), it was found that heat treatment (36–52 °C) caused a similar pattern of denaturation for the two enzyme activities. Furthermore, the *p*-NPPC phosphodiesterase activity and GPC cholinephosphodiesterase activity were found to co-migrate in Sephadex G-150 gel chromatography (Fig. 7). These results suggest that two enzyme activities are present in the same protein molecule. In further support of the above suggestion, the Zn^{2+} requirement was observed commonly for the two phosphodiesterase activities (Table 2).

DISCUSSION

Here, we have described the purification of a phosphodiesterase from mouse brain membrane which catalyses the transformation of *p*-NPPC to *p*-nitrophenol and phosphocholine. The purified enzyme was found to be a kind of glycosylphosphatidylinositol-linked glycoprotein enzyme [18], on the basis of results that the enzyme was effectively (90% yield) solubilized from brain membranes by *B. cereus* PI-phospholipase C, and that the enzyme showed selective binding to a Con A affinity column. The observations that the enzyme appeared as a major protein with a molecular mass of 74 kDa in SDS/PAGE analysis, and that the enzyme activity was found to possess a molecular mass of 75 kDa in gel-permeation h.p.l.c. analysis, indicate that the phosphodiesterase is not dissociable in detergent or high-salt buffer. Although it had been reported previously that simultaneous incubation with EDTA in the presence of substrate had no significant effect on the activity of the partially purified *p*-NPPC phosphodiesterase [7], the present study, using the prior incubation with EDTA, shows that the phosphodiesterase is a metalloenzyme expressing an absolute requirement for Zn^{2+}. The above observation might be explained by the assumption that, once the active site of the enzyme is occupied by substrates, Zn^{2+} ions in the active site are no longer accessible to EDTA. In addition, the phosphodiesterase activity is further characterized by an alkaline optimum pH and covalent binding to membranes. Besides, the enzyme did not cleave phosphodiesters such as *p*-nitrophenylphenylphosphonate, *p*-nitrophenylthymidine and bis(*p*-nitrophenyl) phosphate [7], whereas *p*-NPPC was utilized as a selective substrate for the phosphodiesterase at alkaline pH. Thus the *p*-NPPC phosphodiesterase differed from the previously reported phosphodiesterases [1,2,3,5] or phospholipase C [4,19] in respect of the metal requirement, EDTA sensitivity, optimum pH and association with membranes [1,2,4].

Meanwhile, the purified enzyme was found to convert GPC into phosphocholine and glycerol. The possibility that the hydrolysis of GPC might be due to contamination by microbial PI-phospholipase C activity is excluded, because the purified enzyme is markedly different from microbial phospholipase C, at least in optimum pH and metal requirement. Especially, GPC inhibited competitively the hydrolysis of *p*-NPPC by the phosphodiesterase, and the K_i value of GPC as an inhibitor of the phosphodiesterase was similar to the K_m value of GPC as a substrate for the enzyme. Therefore, it was suggested that the enzyme activities responsible for the hydrolysis of GPC and *p*-NPPC reside in the same enzyme molecule. Further supportive data for the suggestions come from findings that *p*-NPPC phosphodiesterase activity and GPC-hydrolysing activity co-migrate in the chromatographic profiles, and that thermal treatment results in a parallel decrease in both enzyme activities. More convincingly, the re-activation by Zn^{2+} was observed for both enzyme activities and the inhibition by thiols such as cysteamine or thiocholine was similar for the two enzyme activities (D.-E. Sok & M. R. Kim, unpublished work). From these results, it is proposed that the *p*-NPPC phosphodiesterase activity may be due to Zn^{2+}-GPC choline phosphodiesterase. Previously a GPC cholinephosphodiesterase exhibiting an optimum pH of 9.5 and Ca^{2+} activation had been partially purified from the supernatant of rat brain homogenate [10]. However, the properties of the enzyme are much different from those of the phosphodiesterase that we purified, especially in optimum pH, K_m value, metal requirement and association with membranes. Rather, the properties of the purified phosphodiesterase seem to be similar to those of the myelin-bound GPC cholinephosphodiesterase, which was reported to require Zn^{2+} [6]. Although the physiological role of GPC cholinephosphodiesterase was not established clearly, the enzyme may play an important role in the direct supply of phosphocholine for the resynthesis of phosphatidylcholine in subcellular compartments. In the formation of phosphocholine from GPC, Zn^{2+}-requiring GPC cholinephosphodiesterase may be more important than Zn^{2+}-independent GPC cholinephosphodiesterase [8,10], since the activity of the former phosphodiesterase is estimated to be greater than the latter *in vivo* [20]. Recent reports that Zn^{2+}-requiring GPC cholinephosphodiesterase activity was decreased in plaques from autopsy samples from brain tissue from patients with multiple sclerosis [6,21] and that GPC cholinephosphodiesterase activity in brain tissue increases during development suggest that the phosphodiesterase may be directly involved in the homoeostasis of phospholipid metabolism in brain membrane. Further study on the regulation of this phosphodiesterase is expected to reveal further the physiological significance of the enzyme.

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