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Regulation and Inactivation of Brain Phosphocholine-Phosphatase Activity

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Regulation of phosphocholine-hydrolyzing phosphatase (phosphocholine-phosphatase) activity, purified from bovine brain, was examined under physiological conditions. Various endogenous phosphomonoesters, which were utilized as substrate, inhibited the phosphocholine-phosphatase activity competitively (Ki, 5.5-82.0 μM); among phosphomonoesters tested, there was a similar order of capability between the binding affinity of substrate and the inhibitory potency. In addition, phosphate ions also inhibited the phosphatase activity competitively with a Ki value of approximately 167 μM. Although leucine or theophylline inhibited the phosphatase activity at pH 9.0, their inhibitory action decreased greatly at pH 7.4. The pH-Km and pH-Vm profiles indicate that ionizable amino acids are involved in substrate binding as well as catalysis, alluding that the phosphatase activity may be highly dependent on the intracellular pH. Amino acid modification study supports the existence of tyrosine, arginine or lysine residue in the active site, and the participation of tyrosine residue in the catalytic action may be suggested positively from the susceptibiliy to the action of tetranitromethane or HOI-generator. Separately, the oxidative inactivation of phosphocholine-phosphatase activity was investigated. Of oxidants tested, HOONO, HOCl, HOI and ascorbate/Cu^{2+} system were effective to inactivate the phosphatase activity. Noteworthy, a remarkable inactivation was accomplished by 30 μM HOCl in combination with 1 mM KI. In addition, Cu^{2+} (3 μM) in combination with ascorbate at concentrations as low as 0.1-0.3 mM reduced the phosphatase activity to a great extent. From these results, it is proposed that the phosphocholine-phosphatase activity may be regulated endogenously and susceptible to the various oxidant systems *in vivo*.

Key words: Phosphocholine, Phosphatase, Regulation, Oxidants, Inactivation

INTRODUCTION

Metabolism of phosphatidylcholine is initiated by PLA_2-catalyzed removal of acyl group at C-2, which produces lysophosphatidylcholine (Scott *et al.*, 1990). Subsequent removal of acyl group at C-1 of lysophosphatidylcholine generates glycerophosphocholine (Nitsch *et al.*, 1992). In turn, glycerophosphocholine is further hydrolyzed by two types of phosphodiesterases, Zn^{2+}-glycerophosphocholine cholinephosphodiesterase (Kanfer and McCartney, 1989; Sok and Kim, 1992a) and glycerophosphocholine phosphocholinephosphodiesterase (Abra and Quinn, 1976). The hydrolysis of glycerophosphocholine by the former enzyme generates glycerol and phosphocholine, and interestingly, the enzyme is remarkably inhibited by phosphocholine at physiological concentrations (Yuan and Kanfer, 1994). Accordingly, the increase of phosphocholine level may lead to the accumulation of glycerophosphocholine, which was recently observed to promote the aggregation of β–amyloid peptide (Klunk *et al.*, 1997). Previously, it had been reported that there was a remarkable elevation in the level of phosphomonoesters such as phosphocholine in early AD pathogenesis (Pettegrew *et al.*, 1988), and a nearly two- fold increase of glycerophosphocholine level at a later stage (Nitsch *et al.*, 1992). A separate study showed that there was a considerable decrease of phosphocholine-hydrolyzing phosphatase (phosphocholine-phosphatase) activity in temporal regions of AD brains (Kanfer and McCartney, 1986). From these data, it is inferred that the loss of phosphocholine-hydrolyzing phosphatase activity might be related to the increase of phosphocholine level followed by the enhancement of glycerophosphocholine level in brain tissue of AD patients. Meanwhile, the loss of phosphocholine-phosphatase activity may lead to the lowered conversion of phosphocholine to choline, resulting in the decreased supply of choline, a precursor of acetylcholine biosyn-

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thesis. In this respect, it is supposed that the phosphocholine-hydrolyzing phosphatase activity may play a beneficial role in the alleviation of AD symptoms by supplying choline or in the retardation of AD pathogenesis by indirectly reducing the level of glycerophosphocholine. However, the study on the phosphatase, responsible for the hydrolysis of phosphocholine was not been extensively carried out.

Concerning phosphocholine-phosphatase activity, the phosphocholine-specific phosphatase, different from alkaline phosphatase, was reported to reside in the heart tissue (Hatch and Choy, 1987). However, the role of the enzyme in the conversion of phosphocholine to choline is questioned since the enzyme activity at physiological pH is much smaller, compared to alkaline phosphatase. Meanwhile, it was proposed that phosphocholine was a unique substrate for human intestinal alkaline phosphatase, which may be related to the metabolism of phosphocholine (Irino *et al.*, 1994). In addition, it was suggested that alkaline phosphatase may be one of enzymes responsible for the hydrolysis of phosphocholine in other organs such as placenta, liver or bone. Thus, data accumulate that one of important roles of alkaline phosphatase may be related to the metabolism of phosphocholine. Although it had been reported that alkaline phosphatase in various tissues was inhibited by compounds including leucine (Hoylaerts *et al.*, 1992) and theophylline (Farley *et al.*, 1980), most of those inhibition studies was done at alkaline pHs. Furthermore, those studies were performed using an artificial substrate such as p-nitrophenylphosphate, but not phosphocholine, an endogenous substrate. Moreover, there have been no data related to the regulation of phosphocholine-phosphatase activity in brain tissue. Recently (Sok, 1999), it was reported that glycosylphosphatidylinositol (GPI)-anchored alkaline phosphatase, responisble for the hydrolysis of phosphocholine, was susceptible to Cu^{2+}/H_2O_2 system. Subsequenltly (Moon *et al.*, 1999), GPI-anchored alkaline phosphatase was proposed to be transformed to soluble form by GPI-specific phospholipase D in the cytosol of brain tissue. In this study, we extensively examined about the possible regulation of phosphocholine-phosphatase activity and the inactivation process *in vivo* system.

MATERIALS AND METHODS

Materials

Phospholipase C (B. cereus), p-nitrophenylphosphate (p-NPP), phosphocholine, leucine, theophylline, ascorbate, 3-morpholinosydnonimine, sodium benzoate, 4-methyl aminophenol, a-methyl mannoside, concanavalin A sepharose, thiols, phosphomonoesters and chemical modifiers were obtained from Sigma Chemical Co. (St. Louis. MO, USA). Ammonium molybdate, potassium iodide and H_2O_2 (30 %) were from Junsei Chemical Co. (Tokyo, Japan). Metal ions including cupuric sulfate were of analytical grade.

Assay of phosphatase

The phosphocholine-hydrolyzing phosphatase (phosphocholine-phosphatase) activity was determined by incubating the phosphatase in 0.5 ml of 100 mM Tris buffer, pH 7.4 containing 2 mM phosphocholine and 2 mM Mg^{2+} at 38°C for 1 h, unless otherwise described, and the formation of inorganic phosphate was determined according to a modification (Drummond and Yamamoto, 1971) of the procedure of Fiske-Subbarow. Separately, the formation of choline from phosphocholine was measured by the modified method of the enzyme-coupled assay (Masoom *et al.*, 1990; Sok and Kim, 1992b); the reaction mixture was combined with choline oxidase (0.5 unit), peroxidase (590 units), and 1 mM azo-bis(3-ethyl-2,3-dihydroxybenzothiazoline-6-sulfonate). The net absorbance at 450 nm was converted into the amount of choline released. Separately, the phosphatase activity was determined by measuring the amount of p-nitrophenol released from the hydrolysis of p-nitrophenylphosphate (p-NPP) as described before (Brunel and Cathala, 1973); assays were performed in 1 ml of 50 mM Tris buffer, pH 7.4 containing 2 mM p-NPP and 2 mM Mg^{2+} at 38°C. One unit is expressed as the ability of enzyme to hydrolyze one micromole of substrate per min.

Preparation of purified brain phosphatase.

The purification of phosphocholine-phosphatase activity was purified as described previously (Sok, 1999). The extract from phospholipase C treatment, after centrifugation (15,000 g × 20 min), was applied to concanavalin-A sepharose column (2.6 × 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 the above buffer containing 0.25 M a-methyl mannoside (Sok and Kim, 1992b). Next, the enzyme fraction from concanavalin-A sepharose column, after dialysis, as applied to DEAE Sephacel column (2 × 28 cm), which was eluted with 10 mM Tris buffer (pH 7.4) containing a concentration gradient (0 - 0.1 M) of NaCl according to the published procedure (Brunel *et al.*, 1969). The portions possessing the phosphatase activtity were pooled, concentrated and applied to Sephacry S-200 gel column (2 × 50 cm), which was eluted with 10 mM Tris buffer, pH 7.4 containing 0.1 M NaCl. The purified enzyme, which exhibited a relatively homogeneous band with a molecular weight of approximately 92 kDa on SDS-polyacryamide electrophoresis under reducing condition, showed a specific activity of approximately 48 μmole/min. mg protein in the hydrolysis of phosphocholine at pH 7.4. This phosphatase activity is responsible mainly

(>92 %) for the hydrolysis of phosphocholine in the brain homogenate at a neutral pH. The DEAE cellulose or Sephacryl gel chromatography purity was suitable for the present studies.

Hydrolysis of phosphomonoesters by the phosphatase

The hydrolysis of phosphomonoesters by the phosphatase was determined by incubating the phosphatase (approximately 0.1 unit) with each phosphomonoester in 0.5 ml of 100 mM Tris buffer, pH 7.4 containing 2 mM Mg^{2+} at 38°C for 1 h, and the formation of inorganic phosphate was determined as described above. Kinetic parameters, Km and Vm values, were obtained from Lineweaver-Burk plots (Sok and Kim, 1992b).

Effect of pH on kinetic parameters in the hydrolysis of phosphocholine

The enzymatic hydrolysis of phosphocholine was performed in 0.5 ml of 100 mM buffer of various pHs containing 2 mM Mg^{2+} at 38°C for 1 h; pH 7-9 (Tris buffer), pH 9-10 (glycine buffer) and pH 9-12 (carbonate-bicarbonate buffer). Km and Vm values were obtained from Lineweaver-Burk plots.

Inhibition of the phosphocholine-phosphatase activity by phosphomonoesters or other inhibitors

The phosphatase (approximately 0.1 unit) was incubated with phosphocholine of various concentrations in the presence or absence of each phosphomonoester or inhibitor in 0.5 ml of 100 mM Tris buffer, pH 7.4 containing 2 mM Mg^{2+} at 38°C for 1 h, and the formation of choline was determined as described above. The Ki values were determined from Lineweaver-Burk plots. Independently, the phosphatase (approximately 0.1 unit) was incubated with phosphocholine in the presence or absence of each thiol in 0.5 ml of 100 mM Tris buffer, pH 7.4 containing 2 mM Mg^{2+} at 38°C for 1 h, and the formation of phosphate ions was determined as described above.

Inactivation of the phosphocholine-phosphatase activity by chemical modifiers

The phosphatase (0.3 unit) was preincubated with the respective modifier at various concentrations in 0.5 ml of 100 mM Tris buffer (pH 7.4) at 38°C for the time indicated, and the aliquot (20-100 µl) was taken for the assay of remaining activity in the release of phosphate ions from the hydrolysis of phosphocholine (2 mM).

Effect of oxidants on the phosphatase and inactivation of phosphatase by ascorbate/Cu^{2+} system or HOI generator

The phosphatase (0.3 unit) was preincubated with each oxidant system at 38°C in 0.5 ml of 100 mM Tris buffer (pH 7.4) for 5 min, and an aliquot (50 or 100 µl) was taken for the assay of remaining activity in the hydrolysis of phosphocholine as described above. In related studies, the phosphatase was preincubated with ascorbic acid of various concentrations in the presence of 3 µM Cu^{2+} at 38°C in 0.5 ml of 100 mM Tris buffer (pH 7.0) at 38°C, and the aliquot (50-100 µl) was taken for the assay of remaining activity in the hydrolysis of phosphocholine. Separately, the phosphatase (0.3 unit) was preincubated with 30 µM HOCl in the presence of KI in 0.5 ml of 100 mM Tris buffer (pH 7.4) at 38°C, and the aliquot (20-100 µl) was taken for the assay of remaining activity in the hydrolysis of phosphocholine.

RESULTS

Previously (Sok, 1999), it had been reported that alkaline phosphatase purified from brain tissue expressed a phosphocholine-hydrolyzing (phosphocholine-phosphatase) activity. In this study, alkaline phosphatase activity derived from GPI-anchored alkaline phosphatase was found to be responsible for >92 % of total phosphocholine hydrolysis in brain tissue at pH 7.4, indicating that most of phosphocholine-hydrolyzing phosphatase activity in brain tissue may be due to the activity of alkaline phosphatase. Based on this observation, we attempted to figure out the possible regulation of the phosphocholine-hydrolyzing phosphatase (phosphocholine-phosphatase) activity *in vivo* system. First, the role of various endogenous phosphomonoesters as substrates was examined at pH 7.4 (Table I), based on the release of phosphate ions. Lysophosphatidic acid and phosphatidic acid, containing a long acyl chain, were not utilized as substrate. In contrast, phosphomonoesters, devoid of acyl moiety, were hydrolyzed effectively by the enzyme. Overall, there was a similarity of Vm values among phosphomonoesters tested, although the Km values differed according to the alcoholic or phenolic portion of the substrates. In comparison, phosphocholine (Vm, 48 µmole/min.mg protein) seemed to be more readily utilized as substrate than p-nitrophenylphosphate (Vm, 28 µmole/min.mg protein). Phosphocholine exerted an inhibitory action in a competitive manner with a Ki value of 142 µM in the hydrolysis of p-nitrophenylphosphate (data not shown), confirming that two phosphomonoesters were hydrolyzed in the same active site. Similarly, the other endogenous phosphomonoesters inhibited the hydrolysis of p-nitrophenylphosphate by the enzyme in a competitive fashion (data not shown). From these results, it was supposed that endogenous phosphomonoesters could compete with each other in the same active site. When various endogenous phosphomonoesters were tested for the inhibition of the phosphocholine-phosphatase activity at pH 7.4, based

Table I. Kinetic parameters in the hydrolysis of phosphomonoesters and their Ki values

	V max (μmole/min.mg protein)	Km (μM)	Ki (μM)
Lysophosphatidic acid	None	None	-
Phosphatidic acid	None	None	-
Flavin mononucleotide	32.5±7.2	44.2±4.7	5.5±0.5
Pyridoxal phosphate	43.0±7.7	52.3±7.6	9.3±1.2
Thiamine monophosphate	45.7±3.3	87.7±13.2	16.7±2.1
D(-)-3-Phosphoglyceric acid	32.5±2.2	100.0±10.0	82.0±20.3
Phosphocholine	47.9±3.3	72.0±4.3	-
p-Nitrophenylphosphate	28.2±4.5	15.0±1.7	-
Phosphate	-	-	166.7±30.6

-, not determined.The phosphatase (approximately 0.1 unit) was incubated with each phosphomonoester of different concentrations in 0.5 ml of 100 mM Tris buffer, pH 7.4 containing 2 mM Mg^{2+} at 38°C for 1 h and the formation of inorganic phosphate was measured and utilized to determine Km and Vm values as described in Methods. The hydrolysis of p-nitrophenylphosphate was measured, based on the formation of p-nitrophenol. In the inhibition study, the phosphatase (approximately 0.1 unit) was incubated with 2 mM phosphocholine in the presence or absence of each phosphomonoester in the same buffer as above at 38°C for 1 h, and the formation of choline was measured and employed for the determination of Ki values as described in Methods. Values are the mean ± S.D. (n=3).

Table II. Inhibition of phosphocholine-phosphatase activity by thiols or other inhibitors

	Ki (mM)	
	pH 7.4	pH 9.0
Leucine	11.0±3.0	1.83±0.35
Tyrosine	-	5.59±0.51
Theophylline	3.60±1.04	1.18±0.12
Cysteine	3.40±0.53	0.14±0.01
Cysteine methylester	0.01± 0.01	0.01±0.01
N-acetylcysteine	-	1.51±0.37
S-ethyl-L-cysteine	-	15.13±4.50

-, not determined. The phosphatase (approximately 0.1 unit) was incubated with 2 mM phosphocholine in the presence or absence of each thiol at different concentrations in 0.5 ml of 100 mM Tris buffer, pH 7.4 containing 2 mM Mg^{2+} at 38°C for 1 h, and the formation of phosphate ions was measured and utilized to determine Ki values. Separately, the inhibitory action of leucine and theophylline was determined as described above. Values are the mean ± S.D. (n = 3).

on the enzymatic conversion of phosphocholine into choline, all of phosphomonoesters tested showed a competitive inhibition of the phosphocholine-phosphatase activity. In comparison (Table I), flavin monomononucleotide (Ki, 5.5 μM) was more inhibitory than the other phosphomonoesters such as pyridoxal phosphate (Ki, 9.3 μM), thiamine phosphate (Ki, 16.7 mM) or 3-phosphoglyceric acid (Ki, 82 μM). In the following experiments, the hydrolysis products of phosphocholine were examined for the inhibition of the phosphatase; while choline had no effect up to 3 mM, phosphate ions expressed a competitive inhibition with a Ki value of approximately 167 μM. Next, theophylline (Farley *et al.*, 1980) and leucine (Hoylaerts *et al.*, 1992), which had been known to inhibit alkaline phosphatase at alkaline pH, were examined for the ability to inhibit phosphocholine-phosphatase activity at pH 7.4 or pH 9.0 (Table II). Although theophylline (Ki, 1.2 mM) and leucine (Ki, 1.8 mM) inhibited the enzyme competitively and uncompetitively, respectively, at pH 9.0, their inhibitory action at pH 7.4 was much lower. In a separate experiment, thio-analogues were tested for the inhibitory effect on the phosphocholine-phosphatase activity at pH 9.0, since it had been reported that Zn^{2+}-metallohydrolases were sensitive to the action of thiols at alkaline pHs. Table II demonstrates that in contrast to S-ethyl-cysteine, which showed a meager inhibition, cysteine (Ki, 140 μM) and cysteine methylester (Ki, 10 μM) possessed potent inhibitory actions. A modest inhibitory action was exhibited by N-acetyl cysteine. Despite this, the inhibitory action of potent thiol inhibitors, cysteine or cysteine methyl ester, was much lower at pH 7.4. Thus, the ionization of thiol group seemed to be important for the interaction of thiols with the Zn^{2+} site.

In the subsequent experiment, the pH dependence for the hydrolysis or the binding affinity of phospho-

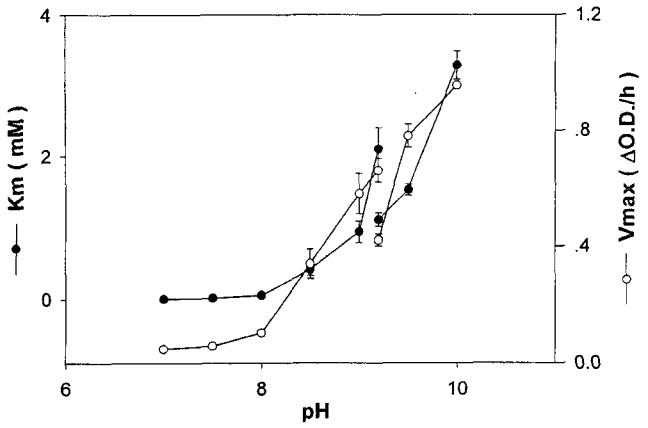

Fig. 1. Effect of pH on kinetic parameters in the hydrolysis of phosphocholine. The enzymatic hydrolysis of phosphocholine was performed in 0.5 ml of 100 mM buffer of various pHs containing 2 mM Mg^{2+} at 38°C for 1 h; pH 7-9 (Tris buffer), pH 9-10 (glycine buffer) and pH 9-12 (carbonate-bicarbonate buffer). Km and Vm values were obtained from Lineweaver-Burk plots. Values are the mean ± S.D. (n=3).

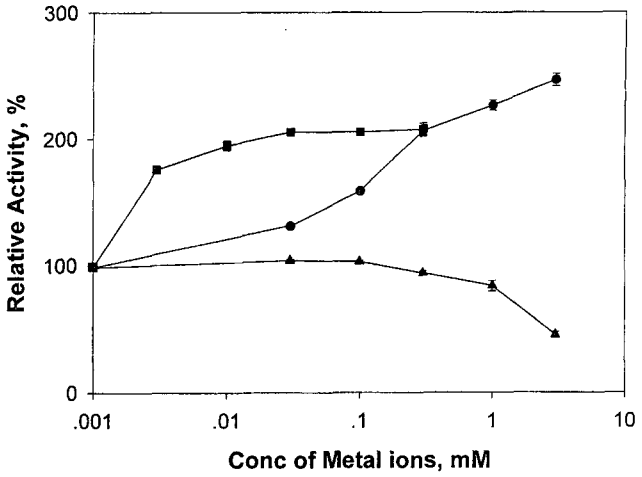

Fig. 2. Effect of divalent metal ions on the phosphocholine-phosphatase activity. The phosphatase (0.1 unit) was incubated with 2 mM phosphocholine in the presence of either divalent metal ion or organic cation at different concentrations in 0.5 ml of 100 mM Tris buffer, pH 7.4 at 38°C for 1 h, and the formation of phosphate ions was measured. Values are the mean ± S.D. (n = 3). ▲ , Cu^{2+}; ■ , Mg^{2+}; ● , Mn^{2+}

choline was examined. Fig 1 shows that the Vm values and Km values differed greatly according to pH. The pH-Km profile (Fig. 1) exhibits a continuous increase of Km value over pH 10, indicating that a basic amino acid residue may be a site for the binding of phosphocholine. Subsequently, the dependence of hydrolytic rate on pH was examined. Fig 1 indicates that the catalytic rate was continuously elevated with increasing pH value up to 10. The Vm value at pHs higher than pH 10 was not measured, since the solubility of phosphocholine at saturating concentrations was limited at pHs higher than pH 10. In a separate experiment, there was a continuous increase of both Km and Vm values up to pH 12 in the hydrolysis of p-nitrophenylphosphate (data not shown). These results may imply that a nucleophilic amino acid residue may be responsible for the hydrolysis of phosphocholine. Since Zn^{2+}-metallohydrolase had been reported to be affected by metal ions, the effect of divalent metal ions on the phosphocholine-phosphatase activity was investigated. Fig 2 indicates that metal ions such as Mg^{2+} or Mn^{2+} expressed an enhancing effect on the phosphocholine-phosphatase activity in a concentration-dependent manner, while Cu^{2+} exerted an inhibitory action at >100 μM. In comparison, Mg^{2+} was more effective than Mn^{2+} at concentrations used. Related analysis demonstrates that Mg^{2+} showed no remarkable change of Km value in the hydrolysis of phosphocholine.

In order to obtain informations about what kind of amino acid residues are involved in the enzymatic reaction, chemical modifications were performed at neutral or alkaline pHs using various chemical modifiers. As Table III demonstrates, iodoacetate, a cysteine or histidine modifier, at 10 mM failed to decrease the enzyme activity

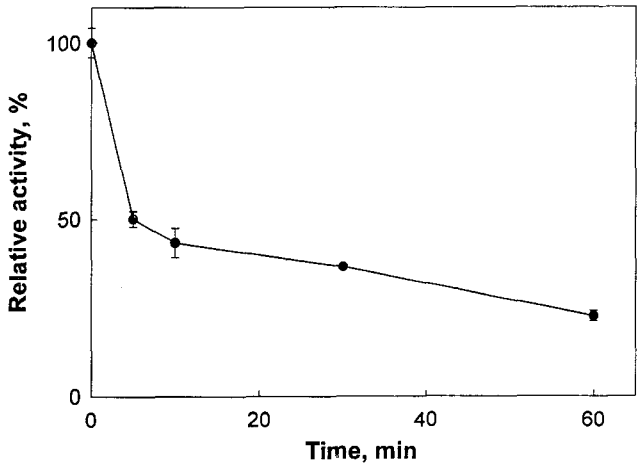

Fig. 3. Time-dependent inhibition of phosphocholine-phosphatase activity by tetranitromethane. The phosphatase (0.3 unit) was preincubated with 100 μM tetranitromethane for various times (0-60 min) in 0.5 ml of 100 mM Tris buffer, pH 9.0 at 38°C, and an aliquot (50-100 μl) was taken for the assay of remaining activity. Data, expressed as a percentage of the control value, are the mean ± S.D. (n=3).

beyond 15%. Meanwhile, modifiers of arginine residue such as 1,2-cyclohexanedione and phenylglyoxal at 10 mM inactivated the enzyme to a great extent (55% and 65 % inactivation, respectively). Moreover, the Km value of phosphocholine decreased from 72 μM to about 48 μM after the exposure to 3 mM phenylglyoxal (data not shown). In addition, lysine modifiers such as trinitrobenzene sulfonic acid or diethyl pyrocarbonate did

inactivate the enzyme to some extent. A remarkable loss of the phosphatase activity was also observed after the exposure to N-acetylimidazlole, a modifier of tyrosine, lysine or cysteine. Besides, either tetranitromethane as a tyrosine modifier or N-bromosuccinimide, a modifier of tyrosine or tryptophan residue, was found to inactivate the enzyme below 1 mM. In further study, it was found that the activity of the phosphatase during preincubation with 0.3 mM tetranitromethane decreased in a time-dependent manner (Fig 3). Noteworthy, the inactivating action of tetranitromethane was much greater at pH 9 than at pH 7.4 (Table III). The inactivation of the phosphatase activity by various tyrosine modifiers led us to assume that the tyrosine residue might be one of amino acids directly involved in the catalysis of the phosphatase.

In an attempt to see the possible oxidative inactivation of the phosphatase *in vivo* system, the enzyme was exposed to various oxidant systems such as H_2O_2, HOCl generator, HOONO generator or HOI generating system, and then the remaining activity was determined based on the formation of phosphate ions from the hydrolysis of phosphocholine as a substrate. Table IV demonstrates that there was a remarkable decrease of phosphatase activity after 1-h exposure to 3-morphlinosydnonimine (0.1 or 1 mM), a peroxynitrite generator, in support of the existence of tyrosine residue in the active site of the phosphatase. In addition, NaOCl, HOCl generator, was found to cause a modest loss of activity in a concentration-dependent manner. The concentration of HOCl required for the effective inactivation was close to the concentration in present in the activated cells (Eiserich, 1998). Of note, the HOCl-induced inactivation was further enhanced in the presence of iodide; in contrast to a slight inactivation (8% inactivation) by 30 μM HOCl alone, the inclusion of iodide greatly enhanced the HOCl-induced inactivation, suggesting that HOI generated from the combination of KI and HOCl was responsible for the inactivation. When the concentration of HOCl was fixed to 30 μM and that of iodide was varied (0.3-10 μM), it was found (Fig. 4) that the phosphatase activity was inactivated in a concentration-dependent manner, and the inactivation by HOCl (30 μM) in combination with KI (1 μM) was demonstrated to be remarkable within 10 min. Separately, metal-catalyzed inactivation of the phosphatase employing ascorbate and Cu^{2+} was examined. As demonstrated in Table IV, ascorbic acid, in combination with Cu^{2+}, caused a great loss (> 73 %) of the phosphocholine-phosphatase activity, whereas some (30 %) inactivation was expressed by 0.3 mM ascorbate alone. When the concentration of ascorbic acid was varied (0.1-1 mM), and that of Cu^{2+} was fixed at 3 μM, it was found (Fig. 5) that a remarkable inactivation was accomplished with ascorbic acid at concentrations as low as 0.1-0.3 mM.

Table III. Effect of amino acid modifier on phosphocholine-phosphatase activity

Treatment	Concentration (mM)	Alkaline phosphatase activity (% of control)
Control		100
Phenylglyoxal	3	76.0±2.3
	10	34.7±3.2
1,2-cyclohexanedione	3	65.5±2.0
	10	44.7±1.4
Iodoacetate	3	95.0±3.7
	10	86.1±1.6
Diethyl pyrocarbonate	3	47.2±2.1
	10	1.6±0.8
N-acetylimidazole	3	77.6±0.9
	10	31.6±1.7
Trinitrobenzen sulfonate	1	60.0±0.5
	3	35.9±2.2
Tetranitromethane	0.3	61.0±0.5
	0.3[1)]	22.5±1.5
N-bromosuccinimide	0.1	52.0±1.7
	0.3	3.8±0.8

[1)] 0.1 M Tris-HCl, pH 9.0. The phosphatase (approximately 0.3 unit) was preincubated for 1 h with the respective modifier at various concentrations in 0.5 ml of 100 mM Tris buffer (pH 7.4) at 38°C for the time indicated, and the aliquot (20-100 μl) was taken for the assay of remaining activity. Data, expressed as a percentage of the control value, are the mean ± S.D. (n=3).

DISCUSSION

Previously (Coleman and Gettins, 1984), it had been

Table IV. Effect of oxidants on phosphocholine-phosphatase activity

Treatment	Concentration	Alkaline phosphatase activity (% of control)
Control		100
H_2O_2	0.3 mM	92.8±2.7
3-Morpholinosydnonimine[1)]	1.0 mM	45.0±0.8
	0.1 mM	76.5±1.2
NaOCl	0.3 mM	79.9±4.5
	0.1 mM	82.9±0.9
	30.0 mM	92.0±2.5
(+KI)	(+ 3 μM)	10.5±0.4
Ascorbic acid	0.3 mM	73.8±0.2
(+Cu^{2+})	(+ 3 μM)	26.3±1.7

[1)] 1 h preincubation. The phosphatase (approximately 0.3 unit) was preincubated with each oxidant system for 10 min or 1 h at 38°C in 0.5 ml of 100 mM Tris buffer (pH 7.4), and an aliquot (50 or 100 μl) was taken for the assay of remaining activity. Data, expressed as a percentage of the control value, are the mean ± S.D. (n=3).

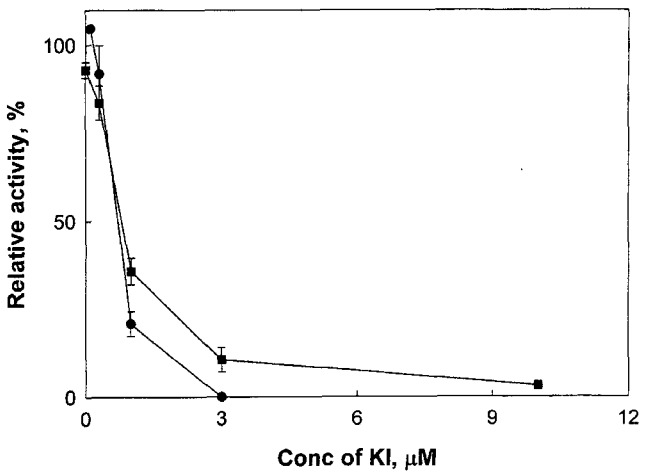

Fig. 4. Inactivation of phosphocholine-phosphatase by HOCl generator in combination with KI. The phosphatase (0.3 unit) was preincubated with 30 mM NaOCl in the presence of KI in 0.5 ml of 100 mM Tris buffer (pH 7.4) at 38 °C, and an aliquot (20-100 ml) was taken for the assay of remaining activity. Data, expressed as a percentage of the control value, are the mean ± S.D. (n=3). ■, 10 min; ●, 30 min preincubation.

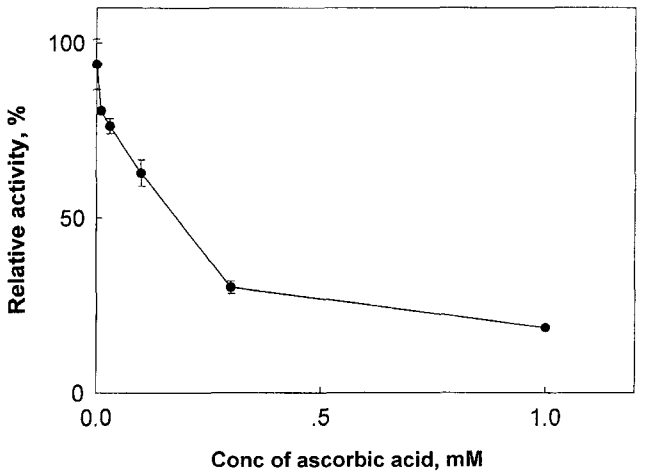

Fig. 5. Inactivation of phosphatase by ascorbic acid/Cu^{2+} system. The phosphatase (0.3 unit) was preincubated with ascorbic acid of various concentrations in 0.5 ml of 100 mM Tris buffer (pH 7.4) containing 3 μM Cu^{2+} at 38°C, and the aliquot (20-100 μl) was taken for the assay of remaining activity in the hydrolysis of phosphocholine. Data, expressed as a percentage of the control value, are the mean ± S.D. (n=3).

reported that the active site of alkaline phosphatase from various sources contained a Zn^{2+} site and cationic binding sites in addition to a nucleophile serine residue. The active site appears to be a non-specific but narrow crevice of a limited size, since phosphomonoesters containing long acyl chains are not proper substrates for this enzyme. Phosphomonoesters, which can be accomodated in the active site, compete with each other to be utilized as a substrate. Overall, endogenous phosphomonoesters showing a higher affinity as a substrate expressed a greater inhibition of the phosphocholine-phosphatase activity at neutral pH. The site for the binding of phosphoryl group will be basic amino acids such as arginine or lysine, since the pH-Km value profile of phosphocholine showed no inflection up to 10. A partial change of Vm or Km value after the exposure of the enzyme to phenylglyoxal, an arginine modifier, might support the idea that the arginine residue, probably responsible for the binding of phosphoryl group, may reside in the active site, consistent with the previous observation (Sun *et al.*, 1999). A remarkable inhibition of the phosphatase by thiols at alkaline pH may be due to their effective association with Zn^{2+} in the active site, in agreement with the previous report on the inhibition of Zn^{2+}-metallohydrolases by thiols (Lennarz and Strittmatter, 1991). In support of the above, the inhibitory potency of cysteine was much greater at pH 9.0 than pH 7.4. The requirement of both thiol group and a-amino group is well evidenced by the failure of N-acetylcysteine or S-ethylcysteine to express a potent inhibition of the phosphatase. All these results are in support of the existence of Zn^{2+} site as an additional site for the binding of phosphoryl group of substrate. Earlier observers (Coleman and Gettins, 1984; Sun *et al.*, 1999) showed that the catalytic site of the phosphatase included the serine residue, which was involved in the primary phosphorylation step. Also, it is reaffirmed that at alkaline pH, the release of phosphate ions from the non-covalent enzyme-phosphte complex determines the rate, whereas at acidic pH the hydrolysis of the covalent enzyme-phosphate complex controls the rate. The possible participation of the nucleophile amino acid residue in the catalysis is suggested from the pH-rate profile of titration curve, similar to that in the hydrolysis of p-nitrophenylphosphate by kidney alkaline phosphatase (Cathala *et al.*, 1975). Present data implies that the tyrosine residue may also be one of amino acids involved in the catalysis. A positive support for this notion is derived from the observation that the phosphatase is sensitive to the inactivation by tetranitromethane, a tyrosine-specific modifier (Page and Wilson, 1985). This may be further supported from the great difference in the sensitivity to tetranitromethane between pH 7.4 and pH 9.0. Furthermore, the enzyme was also inactivated by N-acetylimidazole and N-bromosuccinimide, modifiers of tyrosine residue. Additional support may come from another observation that the enzyme is inactivated during the incubation with HOI-generating system, another tyrosine modifier (Sun and Dunford, 1993). These results may support the idea that the tyrosine residue might also participate in the catalysis, contrary to a previous report (Christen *et al.*, 1971) that the tyrosine residue might not be involved in the catalysis of alkaline phosphatase

activity, based on a partial inactivation (>40%) by tetranitromethane. Although this discrepancy can not be explained by present results, it might be due to the different source of alkaline phosphatase or the different pH used. Despite the participation of nucleophile amino acid in catalysis, the greater hydrolysis of phosphocholine, compared to p-nitrophenylphosphate, is not consistent with the electronic effect of substituents on the nucleophilic hydrolysis. Therefore, it is reaffirmed that the attack of substrate by nucleophile amino acid residue may not be a rate-determining step at neutral pH. Instead, it is supposed that the rate-determining step of phosphocholine-phosphatase activity at physiological pH may be the release of phosphate ions. In this relation, it is possible to postulate that Mg^{2+} may enhance the activity of phosphocholine-phosphatase by reducing the affinity of phosphate ions. This notion might be consonant with the earlier report that alkaline phosphatase showing an absolute requirement for Zn^{2+} was further activated by Mg^{2+} (Janeway *et al.*, 1993).

Concerning the possible negative regulation of the phosphatase in brain tissue, the most probable endogenous compounds to affect the phosphatase activity *in vivo* system could be phosphomonoesters or phosphate ions, which showed a remarkable inhibition of phosphocholine-phosphatase activtiy. Although the inhibitory concentration of some phosphomonoesters appears to be higher than their physiological concentrations, it is conceivable that total concentration of inhibitory phosphomonoesters *in vivo* sytstem may be high enough to affect the phosphatase activity, which needs a further study in biological relevance. Another compound capable of inhibiting the phosphocholine-phosphatase activity at physiological pH may be phosphate ions. The inhibitory concentration (Ki value) of phosphate ions at pH 7.4 is below the physiological concentration (Devlin, 1992). The actual inhibition of the phosphatase by endogenous thiols such as cysteine or glutathione is not likely under the physiological condition, since the inhibitory concentration of these thiols was higher than the physiological concentration at neutral pH. In addition, the inhibitory action of amino acids and theophylline, which had been well known to inhibit alkaline phosphatase at alkaline pHs, might not be significant at physiological pH. Besides, the pH of brain tissue or cells appears to be one of important factors to affect the phosphocholine-phosphatase activity, since the phosphocholine-phosphatase activity was enhanced continuously as the pH was increased from 7.0 to 10.0. Therefore, a small change of pH *in vivo* system is supposed to alter the phosphatase activity to a remarkable extent. A positive regulator would be Mg^{2+}, which exerted an activatory actions at millimolar concentrations, close to a physiological concentration (Devlin, 1992). The activatory effect of Mg^{2+} may be beneficial for the phosphocholine-phosphatase activity, along with its stabilizing effect (Janeway *et al.*, 1993).

The present study on the inactivation of the phosphocholine-phosphatase activity indicates that the phosphatase is one of enzymes susceptible to attack by various biological oxidants; noteworthy, the active site of phosphocholine-phosphatase activity is suggested to contain a tyrosine residue, which is well known to be susceptible to HOCl, HOONO or hydroxyl radicals (Winterbourn *et al.*, 1997; Yim *et al.*, 1996; Eiserich *et al.*,1998; Kim and Kwon, 1999). Since there are many evidences on the generation of oxidants such as H_2O_2, HOCl, or HOONO in brain cells (Packer, 1995; Ames *et al.*, 1993; Bagasra *et al.*, 1995; Yim *et al.*, 1996; Eiserich *et al.*,1998), it is highly likely that the phosphocholine-phosphatase may be susceptible to the oxidative inactivation in brain tissue. Earlier reports on the loss of the phosphatase in temporal regions of brain of AD patients might be explained in part by our observation. The phosphatase activity was quite susceptible to the oxidation by hydroxyl radicals generated from Cu^{2+}/ascorbate system, in support of the previous observation (Sok, 1999) that the phosphocholine-hydrolyzing activity was inactivated by Cu^{2+}/H_2O_2 system. In comparison, Cu^{2+}/ascorbate system appeared to be more potent than Cu^{2+}/H_2O_2 system in inactivating the phosphatase. This might be the facilitated redox cycle of copper ions in the presence of ascorbic acid. Furthermore, there was a report on the selective association of ascorbic acid with alkaline phosphatase, which was evidenced by fluorescence study (Martorana *et al.*, 1986). In neurodegenerative diseases, where there is an increased release of transition metal ions, the hydroxyl radicals-mediated oxidative inactivation of the phosphatase would be more probable. Also, the enzyme was inactivated by HOONO-generating system, consistent with the notion that the tyrosine residue participates in the catalysis of the phosphatase. Since there is growing evidences of HOONO generated from the combination of superoxide anion and nitric oxide in age-related brain diseases (Ames *et al.*, 1993; Bagasra *et al.*, 1995; Good *et al.*, 1996; Yim *et al.*, 1996), it is possible to suppose that peroxynitrite may be one of oxidants responsible for the oxidative loss of the phosphatase activity *in vivo* system. Interestingly, the phosphatase was inactivated partially by HOCl (100-300 μM) alone, and moreover, a remarkable inactivation by HOCl at a low concentration (30 μM) was achieved in the presence of iodide at submicromolar or micromolar concentrations. Thus, HOCl either alone or in combination with iodide is supposed to cause the inactivation of the phosphatse activity. Therefore, It is possible to surmise that the excessive generation of HOCl by myeloperoxidase (Winterbourn *et al.*, 1997; Eiserich *et al.*, 1998) *in vivo* system might lead to the gradual decrease of phosphatase activity in organs exposed to iodide of low concentrations.

Taken all together, it is proposed that the phosphatase may be regulated by endogenous compounds or oxidative inactivation process. Despite this possibility, the actual inactivation of the phosphatase *in vivo* system will differ according to the concentration of various protectors, such as antioxidants or antioxidant enzymes (Yim *et al.*, 1994), as well as the physiological ligands of the phosphatase. In addition, the extent of oxidative stress in brain tissue will be another factor. In a preliminary experiment (data not shown), there was no remarkable loss of phosphocholine-phosphatase activity in brain tissue of mice exposed to N-metyl-D-aspartic acid at a dose, which induced a mild oxidative damage. Further study employing a chronic oxidative damage in a specific region of brain tissue is to be followed to evaluate the susceptibility of phosphocholine-phosphatase to oxidative damage *in vivo* system.

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