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Novel phosphoserine phosphatase inhibitors

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Abstract

Phosphoserine phosphatase (EC 3.1.1.3) catalyzes the final step in the major pathway of L-serine biosynthesis in brain. This enzyme may also regulate the levels of glycine and D-serine, the known and putative co-agonists for the glycine site of the *N*-methyl-D-aspartate receptor in caudal and rostral brain regions, respectively. Using L-phosphoserine as substrate, the rank order potency for inhibition of phosphoserine phosphatase was *p*-chloromercuriphenylsulfonic acid (CMPSA) > glycerophosphorylcholine ≫ hexadecylphosphocholine ≥ phosphorylcholine > *N*-ethylmaleimide ≥ L-serine > fluoride > D-2-amino-3-phosphonopropionic acid (D-AP3). Glycerylphosphorylcholine (IC_{50} 18 μM) was found to be an uncompetitive inhibitor of phosphoserine phosphatase. Glycerylphosphorylcholine probably binds a novel site on the enzyme since the known allosteric inhibitor L-serine is highly selective for its feedback regulatory site, indicated by the inactivity of 25 L-serine analogs. Fluoride ion (IC_{50} 770 μM) may bind the active site as has been shown for other Mg^{2+}-dependent enzymes. The sulfhydryl reagent CMPSA is a potent, noncompetitive inhibitor of the enzyme using L-phosphoserine as substrate (IC_{50} 9 μM) but is > 300-fold less potent using D-phosphoserine as substrate. Substrate-dependent differences are also observed with the sulfhydryl alkylator *N*-ethylmaleimide, which inhibits L-phosphoserine, but stimulates D-phosphoserine hydrolysis. These sulfhydryl reagents may dissociate multimeric forms of the enzyme to form monomers; the multimeric forms and monomers may preferentially cleave L- and D-phosphoserine, respectively. Phosphorylcholine esters and sulfhydryl reagents may prove useful in determining the contribution of phosphoserine phosphatase to the biosynthesis of glycine and D-serine in neuronal tissue in vitro. © 1997 Elsevier Science B.V.

Keywords: Phosphoserine phosphatase; L-Serine; D-Serine; Glycerylphosphorylcholine; Phosphorylcholine; *p*-Chloromercuriphenylsulfonic acid; *N*-Ethylmaleimide

1. Introduction

D-Serine is a selective agonist at the glycine co-agonist site of the *N*-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors (Wood, 1995). The presence of this D-amino acid has been convincingly demonstrated in rat (Hashimoto et al., 1992, 1995) and human (Chouinard et al., 1993; Hashimoto et al., 1993; Kumashiro et al., 1995) brain. Although critical questions remain regarding the synaptic levels of D-serine as well as its synthesis, release and uptake, several authors have postulated that D-serine is an endogenous ligand for the 'glycine' site of the NMDA receptor, particularly in more rostral brain structures (Chouinard et al., 1993; Hashimoto et al., 1992, 1993, 1995; Kumashiro et al., 1995). Elucidation of the mechanisms regulating synaptic D-serine levels will be critical in determining the impact of endogenous D-serine on brain excitability.

Although the biosynthesis of D-serine could involve a novel biochemical pathway, known biosynthetic routes leading to L-serine may be the source of D-serine. Potential sources of D-serine include epimerization of L-serine, formation from L-phosphoserine during hydrolysis by phosphoserine phosphatase, or from glycine via the glycine cleavage system (Fig. 1). Since other D-amino acids are known to exist in brain (e.g., D-aspartate) (Hashimoto et al., 1993), the presence of specific racemases or epimerases in mammalian brain which would catalyze the epimerization of free L-amino acids is an attractive concept, although, to our knowledge, no free amino acid racemase or epimerase is known to exist outside of bacteria and yeast. However, posttranslational modification of proteins resulting in conversion of L- to D-amino acid residues is known to occur in certain vertebrate species (Kreil, 1994). Recently, the findings of reduced D-serine levels in post-

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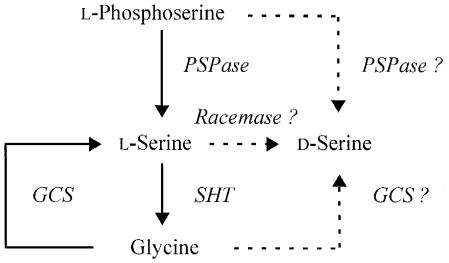

Fig. 1. Possible biosynthetic routes for D-serine. PSPase, phosphoserine phosphatase (EC 3.1.3.3); SHT, serine hydroxymethyl transferase (EC 2.1.2.1); CGS, glycine cleavage system (EC 1.4.3.3).

mortem brain tissue from patients lacking the glycine cleavage system and in rats treated with the glycine cleavage system inhibitor cysteamine have implicated the glycine cleavage system as a potential metabolic source of D-serine (Iwama et al., 1997).

Phosphoserine phosphatase (EC 3.1.3.3) catalyzes the hydrolysis of L-phosphoserine to form L-serine and inorganic phosphate in the final step of the phosphorylated pathway of L-serine biosynthesis, which accounts for greater than 90% of L-serine in brain (Bridgers, 1965) (Fig. 2). Phosphoserine phosphatase is a Mg^{2+}-dependent enzyme having an estimated molecular weight of 47–65 kDa (Bridgers, 1969; Paoli et al., 1974), although this may represent a dimeric form of the enzyme with a monomeric molecular weight of 26 kDa (Moro-Furlani et al., 1980). The *serB* gene thought to encode phosphoserine phosphatase has been cloned from *E. coli* and predicts a protein of 322 amino acids with a molecular weight of 35 kDa (Neuwald and Stauffer, 1985).

In rat brain synaptosomes, phosphoserine phosphatase cleaves L-phosphoserine resulting in L- and D-serine formation (Wood et al., 1996), although D-serine may be formed during or subsequent to L-phosphoserine hydrolysis. Whereas phosphoserine phosphatase can cleave D-phosphoserine to form D-serine in vitro, D-phosphoserine does not exist in brain (Goodnough et al., 1995). The neuronal source of D-serine indicated by synaptosome studies using gas chromatographic-mass spectrometry detection (Wood et al., 1996) contrasts with the reported glial localization using immunocytochemical techniques (Schell et al., 1997).

Since phosphoserine phosphatase appears to be critical for the biosynthesis of D-serine, specific inhibitors of this enzyme are expected to provide useful tools to explore the contribution of endogenous D-serine to NMDA receptor function. The present study evaluates numerous compounds as potential inhibitors of phosphoserine phosphatase and several novel inhibitors are presented.

2. Materials and methods

2.1. Chemicals

O-Acetyl-L-serine, L-alanine, L-aspartate, azaserine (*O*-diazoacetyl-L-serine), *N*-*t*-butyloxycarbonyl-L-serine (*N*-*t*-BOC-L-serine), *O*-*t*-butyl-L-serine, choline chloride, *p*-

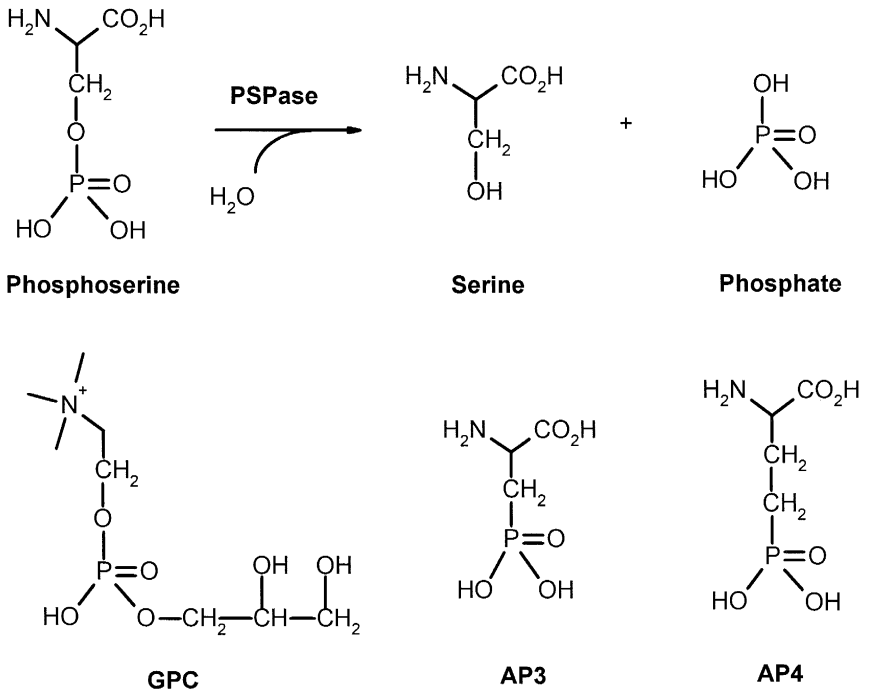

Fig. 2. Reaction catalyzed by phosphoserine phosphatase (PSPase) and structures of the inhibitors L-α-glycerophosphorylcholine (GPC) and 2-amino-3-phosphonopropionic acid (AP3) and the structural analog 2-amino-4-phosphonobutyric acid (AP4).

chloromercuriphenylsulfonic acid (CMPSA), chlorpromazine, β-cyano-L-alanine, L-cysteine, *N*-dansyl-D,L-serine, dithiothreitol, *N*-ethylmaleimide, L-α-glycerophosphorylcholine, hexadecylphosphocholine, D,L-homoserine, iodoacetic acid, D,L-isoserine, α-methyl-D,L-serine, *O*-methyl-D,L-serine, D,L-*threo*-β-phenylserine, L-serinamide, D-serine, L-serine, L-serine ethyl ester, D,L-serine hydroxamate, L-serine-*O*-sulfate, serinol (2-amino-1,3-propanediol) and trifluoperazine were obtained from Sigma (St. Louis, MO, USA). D,L-2-Amino-5-phosphonopentanoic acid (D,L-AP5), D,L-2-amino-6-phosphonohexanoic acid (D,L-AP6) and D,L-2-amino-7-phosphonoheptanoic acid (D,L-AP7) were from Tocris Cookson (St. Louis, MO, USA). D,L-2-Amino-4-phosphonobutyric acid (D,L-AP4) was from Research Biochemicals International (Natick, MA, USA). *N*-Benzoyloxycarbonyl-L-serine (*N*-CBZ-L-serine; *Z*-Ser-OH), L-phosphoserine and D-phosphoserine were from Bachem (Torrance, CA, USA). L-Cycloserine, phosphorylcholine and sodium fluoride were from Aldrich (Milwaukee, WI, USA). D-Cycloserine was from Fluka (Ronkonkoma, NY, USA). β-Chloro-D,L-alanine was from ICN (Costa Mesa, CA, USA). *O*-Benzyl-L-serine (H-Ser-(Bzl)-OH) was from Nova Biochem (San Diego, CA, USA).

2.2. Partial purification of phosphoserine phosphatase

Phosphoserine phosphatase was partially purified according to the method of Bridgers (1967) as described previously (Hawkinson et al., 1996). Approximately 22 g frozen rat brain cortex (ABS, Wilmington, DE) was homogenized in ice-cold acetone (approx. 3 g cortex/20 ml) and centrifuged at $6600 \times g$ for 20 min at 4°C. The pellets were resuspended in ice-cold acetone (6 g wet weight cortex/10 ml), homogenized, and centrifuged at $6600 \times g$ for 20 min at 4°C. The pellet was dried under nitrogen to form the acetone powder, which was resuspended and homogenized in Tris–Mg buffer (10 mM Tris–HCl/10 mM $MgCl_2$/1 mM EDTA, pH 7.5) (approx. 22 g original wet weight cortex/50 ml), stirred on ice for 30 min, and centrifuged at $18{,}400 \times g$ for 35 min. The pellet was resuspended in Tris–Mg buffer (approx. 22 g original wet weight cortex/17 ml) and centrifuged at $18\,400 \times g$ for 35 min. The supernatants were combined to produce the acetone powder extract. Ammonium sulfate (13.73 g) was slowly added to 57 ml acetone powder extract on ice and stirred for 30 min. The suspension was centrifuged at $7200 \times g$ for 30 min at 4°C, the pellet was discarded, and 9.49 g ammonium sulfate was added to 57 ml supernatant and stirred overnight on ice. The suspension was centrifuged at $7200 \times g$ for 1 h at 4°C, the supernatant was discarded, and the pellet was resuspended in 2 ml Tris–Mg buffer and stored at −80°C.

2.3. Phosphoserine phosphatase assay

The phosphoserine phosphatase assay was described previously (Hawkinson et al., 1996). The thawed, partially purified phosphoserine phosphatase preparation was diluted in 50 mM 2-(*N*-morpholino)ethanesulfonic acid (MES)/5 mM $MgCl_2$, pH 6.2 (MES-Mg buffer) and 150 μl aliquots containing 40–50 μg protein were incubated with 50 μl substrate in MES–Mg buffer for 30 min at 37°C (final volume 250 μl). In inhibition experiments, D- or L-phosphoserine was used as substrate at a concentration of 500 or 100 μM, respectively. Test compounds were

Table 1
Inhibition of rat brain phosphoserine phosphatase using L- or D-phosphoserine as substrate

Inhibitor	L-Phosphoserine			D-Phosphoserine		
	IC_{50} (μM)	I_{max} (%)	Hill number	IC_{50} (μM)	I_{max} (%)	Hill number
CMPSA	9.3 ± 0.4	71 ± 2	2.5 ± 0.6	2900 ± 400	99 ± 3 [b]	1.9 ± 0.4
Glycerophosphorylcholine	18 ± 1	104 ± 2	4.5 ± 0.5	31 ± 2	103 ± 1	2.29 ± 0.04
Hexadecylphosphocholine	220 ± 10	91 ± 3	2.1 ± 0.2	180 ± 20	97 ± 5	2.3 ± 0.2
Phosphorylcholine	225 ± 6	96 ± 8	1.4 ± 0.2	990 ± 140	107 ± 6	1.24 ± 0.05
N-ethylmaleimide	430 ± 40	55 ± 3	1.2 ± 0.2	760 ± 70 [e]	58 ± 6	0.83 ± 0.16
L-Serine [a]	460	81	1.05	590	79	1.1
Chlorpromazine [c]	600 ± 20	100 ± 1 [b]	1.1 ± 0.1	550 ± 20	102 ± 1 [b]	1.21 ± 0.04
Fluoride	770 ± 70	84 ± 6	2.20 ± 0.02	390 ± 30	99 ± 5	2.5 (1.4–3.5) [d]
D-AP3 [a]	990	96	0.82	118	94	0.76
Trifluoperazine [c]	1300 ± 120	98 ± 3 [b]	1.1 ± 0.2	1000 ± 300	102 ± 4	1.2 ± 0.1
D,L-AP3 [a]	1400	101	0.82	190	94	0.75
L-AP3 [a]	2090	104	0.86	370	98	0.73

[a] Values from Hawkinson et al. (1996).
[b] Extrapolated.
[c] Not corrected for inhibition of inorganic phosphate-generated color formation.
[d] 95% confidence interval.
[e] EC_{50}, E_{max} and Hill values for enhancement of substrate cleavage. Partially purified enzyme incubated with 100 μM L-phosphoserine or 500 μM D-phosphoserine as substrate for 30 min at 37°C in the presence of increasing concentrations of inhibitor in duplicate. Values are means ± S.E.M. of at least three independent experiments.

added in 50 μl MES-Mg buffer at a final concentration of 1 mM. Active inhibitors were evaluated at 9 concentrations as indicated in the figures. In saturation experiments, increasing concentrations of L-phosphoserine were used in the absence or presence of 20 μM CMPSA or 15 μM glycerophosphorylcholine. The incubations were terminated by addition of 250 μl 1 M HCl and centrifuged on a microfuge at maximal rpm for 10 min. Protein concentration was determined using a modified Lowry procedure (Peterson, 1977).

2.4. Determination of inorganic phosphate

Inorganic phosphate was measured colorimetrically in 96-well polystyrene plates using the method of Fisher and Higgins (1994) as described previously (Hawkinson et al., 1996). Aliquots (100 μl) of the terminated incubation supernatant was mixed with 100 μl malachite green reagent. The malachite green reagent was prepared just prior to use and consisted of 10 ml water, 2 ml 1.3 mM malachite green in 3.6 M H_2SO_4, 0.5 ml 7.5% ammonium molybdate and 40 μl 11% Tween 20. The standard curve consisted of 0–6 nmol inorganic phosphate added in 100 μl 1:1 MES–Mg buffer:1 M HCl. The plate was allowed to stand for 2 h at RT and the absorbance at 650 nm determined using a microtiter plate spectrophotometer (Molecular Devices). Controls for all active inhibitors included direct addition of inhibitor to the malachite green reagent in the absence and presence of 3 nmol inorganic phosphate to measure color formation due to the compound itself and inhibition of phosphate-generated color formation, respectively. For compounds containing a hy-

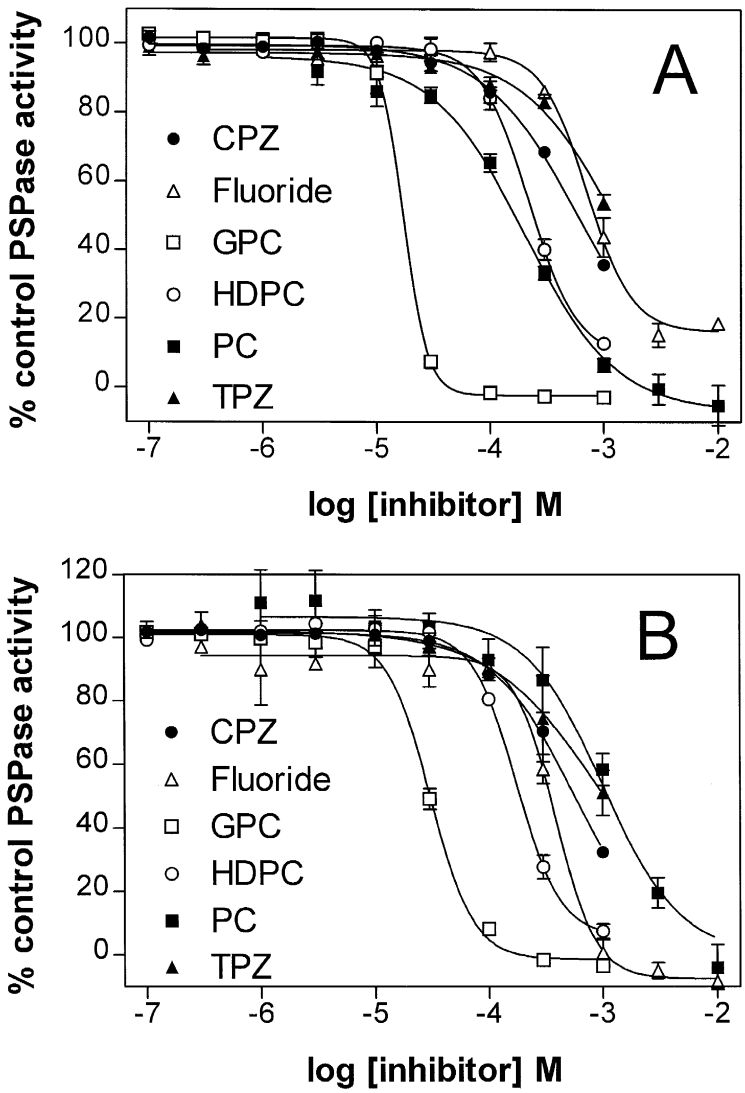

Fig. 3. Inhibitors of rat brain phosphoserine phosphatase. (A) L-Phosphoserine (100 μM) as substrate. (B) D-Phosphoserine (500 μM) as substrate. CPZ, chlorpromazine; GPC, L-α-glycerophosphorylcholine; HDPC, hexadecylphosphocholine; PC, phosphorylcholine; TPZ, trifluoperazine. Partially purified enzyme incubated with substrate for 30 min at 37°C in the presence of increasing concentrations of inhibitor. The calculated IC_{50}, I_{max} and Hill values are shown in Table 1.

drolyzable phosphate group, an additional control consisted of an enzyme reaction in the absence of phosphoserine substrate to measure potential hydrolysis of the inhibitor.

2.5. Estimation of interatomic distances

The distances between the β-carbon and the phosphorus atom of AP4 and phosphoserine were calculated from published crystal structures (Putkey and Sundaralingam, 1970; Chekhlov, 1992). For AP3, this distance was estimated for the energy minimized conformation using Chem3D 3.2 (CambridgeSoft).

2.6. Data analysis

For inhibition experiments, the mean ± S.E.M. percent inhibition of enzyme activity at a fixed concentration of test compound was calculated from at least three independent experiments. For active inhibitors, the concentration producing 50% inhibition of enzyme activity (IC_{50}), the maximal extent of inhibition (I_{max}) and the Hill value were

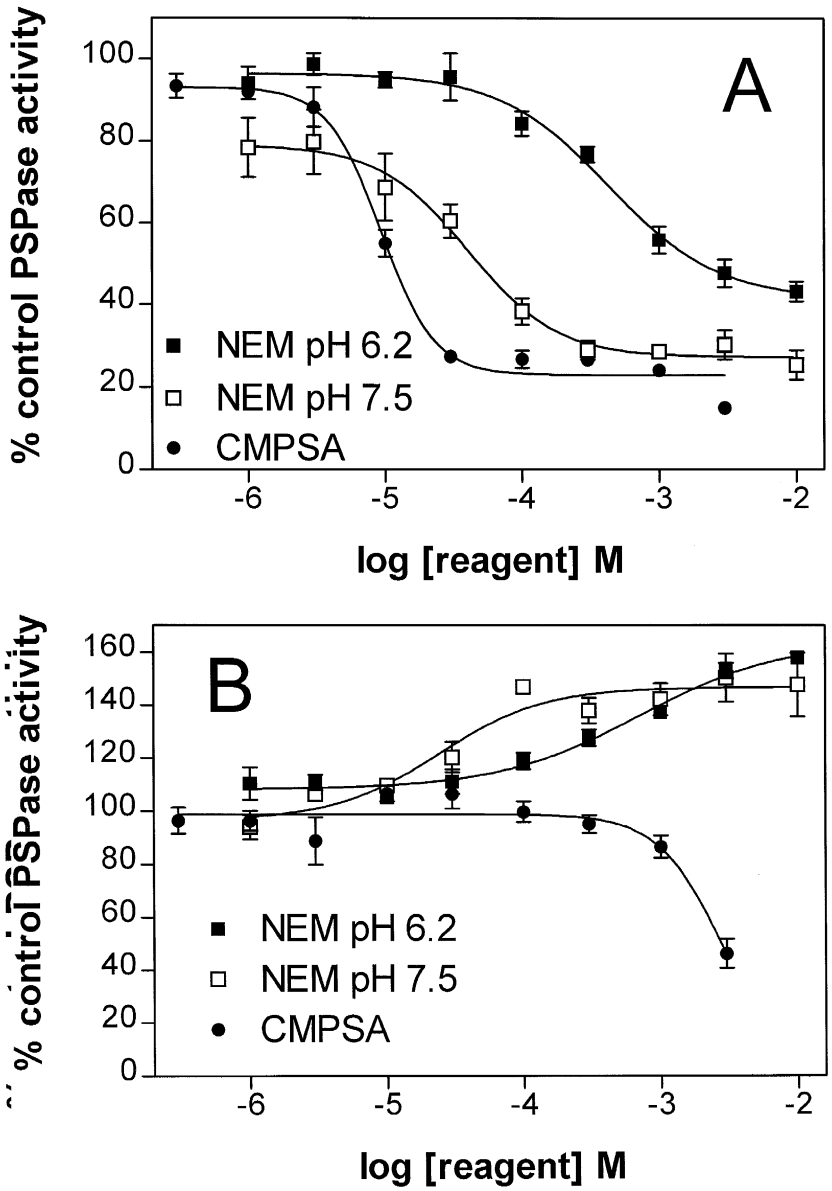

Fig. 4. Effect of sulfhydryl reagents on rat brain phosphoserine phosphatase. (A) L-Phosphoserine (100 μM) as substrate. (B) D-Phosphoserine (500 μM) as substrate. NEM, *N*-ethylmaleimide; CMPSA, *p*-chloromercuriphenylsulfonic acid. Partially purified enzyme incubated with substrate for 30 min at 37°C in the presence of increasing concentrations of inhibitor. The incubations in the presence of *N*-ethylmaleimide were at pH 6.2 (standard assay) or at pH 7.5 as indicated. The calculated IC_{50}, I_{max} and Hill values for the standard assay conditions (pH 6.2) are shown in Table 1. At pH 7.5, *N*-ethylmaleimide inhibited phosphoserine phosphatase activity using L-phosphoserine as substrate with an IC_{50} of 42 ± 11 μM, an I_{max} of 53 ± 9% and a Hill value of 1.5 ± 0.4. At pH 7.5, *N*-ethylmaleimide stimulated phosphoserine phosphatase activity using D-phosphoserine as substrate with an EC_{50} of 32 ± 14 μM, an E_{max} of 56 ± 17% and a Hill value of 1.6 ± 0.5.

estimated from 9 point concentration inhibition data using the sigmoidal equation in Prism 2.0 (GraphPad). For saturation experiments, the Michaelis constant (K_m) and maximal velocity (V_{max}) were estimated using the hyperbolic equation in Prism. This hyperbolic fit was transformed as the double-reciprocal and plotted along with the transformed data to construct the Lineweaver–Burke plot. IC_{50}, I_{max}, Hill slope, K_m and V_{max} values are expressed as the mean ± S.E.M. of individual experiments.

3. Results

3.1. Potency of phosphoserine phosphatase inhibitors

Using L-phosphoserine as substrate, the most potent inhibitor of phosphoserine phosphatase was the organic mercurial reagent *p*-chloromercuriphenylsulfonic acid (CMPSA) (IC_{50} 9.3 μM) (Table 1). The rank order potency for inhibition was CMPSA > glycerophosphorylcholine ≫ hexadecylphosphocholine ≥ phosphorylcholine > *N*-ethylmaleimide ≥ L-serine > chlorpromazine > fluoride > D-2-amino-3-phosphonopropionic acid (D-AP3) > trifluoperazine ≥ D,L-2-amino-3-phosphonopropionic acid (D,L-AP3) > L-2-amino-3-phosphonopropionic acid (L-AP3) (Table 1, Fig. 3A, Fig. 4A). Using D-phosphoserine as substrate, the most potent inhibitor of phosphoserine phosphatase was glycerophosphorylcholine (IC_{50} 31 μM) (Table 1). The rank order potency for inhibition of phosphoserine phosphatase was glycerophosphorylcholine > D-AP3 > hexadecylphosphocholine ≥ D,L-AP3 > L-AP3 ≥ fluoride > chlorpromazine ≥ L-serine > phosphorylcholine ≥ trifluoperazine > CMPSA (Table 1, Fig. 3B, Fig. 4B). The activity of the choline esters (glycerophosphorylcholine, hexadecylphosphocholine and phosphorylcholine) was not simply due to the presence of choline, since choline chloride itself was inactive (Table 2). Appropriate controls indicated that none of the active inhibitors generated color or inhibited phosphate-generated color formation. In addition, the choline esters, which contain a hydrolyzable phosphate group, were not hydrolyzed to form inorganic phosphate.

Whereas D- and L-AP3 were active phosphoserine phosphatase inhibitors (Hawkinson et al., 1996), other amino acid phosphonates (D,L-AP4, D,L-AP5, D,L-AP6, D,L-AP7) were inactive (Table 2). Chlorpromazine and trifluoperazine were relatively weak inhibitors (IC_{50} ~ 600 μM and ≥ 1 mM, respectively); these values were overestimates due to the inhibition of color formation by these compounds. Similar weak inhibition by trifluoperazine and chlorpromazine has been reported previously (Veeranna and Shetty, 1991).

Several other compounds were found to produce < 20% inhibition at a concentration of 1 mM (Table 2). L-Serine analogs containing an elongated side chain (D,L-homoserine), the hydroxy-amino regioisomer (D,L-isoserine) and the enantiomer (D-serine) were inactive. Amino acids lacking the β-hydroxyl group (L-alanine, L-aspartate, L-cysteine, β-chloro-D,L-alanine and β-cyano-L-alanine) and analogs containing a derivatized β-hydroxyl group (*O*-acetyl-L-serine, azaserine, *O*-benzyl-L-serine, *O*-*t*-butyl-L-serine, *O*-methyl-D,L-serine and L-serine-*O*-sulfate) were inactive. Analogs containing a derivatized α-amino group (*N*-*t*-BOC-L-serine, *N*-CBZ-L-serine and *N*-dansyl-D,L-serine) were also inactive. Similarly, analogs containing a

Table 2
Compounds inactive as inhibitors of phosphoserine phosphatase (< 20% inhibition at 1 mM) using L- or D-phosphoserine as substrate

Compound (1 mM)	Inhibition (%)	
	L-phosphoserine	D-phosphoserine
Amino acids		
L-Alanine	6.1 ± 2.1	1.7 ± 1.2
L-Aspartate	3.3 ± 1.0	1.3 ± 3.9
L-Cysteine	−1.0 ± 2.6	1.9 ± 0.3
D,L-Homoserine	−0.2 ± 1.5	2.9 ± 1.4
D,L-Isoserine	−0.1 ± 1.1	0.3 ± 2.0
D-Serine	2.4 ± 3.6	3.9 ± 2.9
Serine derivatives		
O-Acetyl-L-serine	4.0 ± 2.7	2.8 ± 3.5
Azaserine (*O*-diazoacetyl-L-serine)	2.2 ± 1.2	−0.8 ± 3.2
O-Benzyl-L-serine	3.1 ± 1.5	3.6 ± 4.7
N-*t*-BOC-L-Serine	−0.1 ± 3.6	0.9 ± 3.4
O-*t*-Butyl-L-serine	2.0 ± 2.4	2.2 ± 2.5
N-CBZ-L-Serine	−1.2 ± 2.0	2.0 ± 2.5
N-Dansyl-D,L-serine	1.7 ± 3.4	0.6 ± 3.5
α-Methyl-D,L-serine	5.1 ± 3.0	5.8 ± 1.4
O-Methyl-D,L-serine	1.0 ± 3.8	1.5 ± 0.4
D,L-*threo*-β-Phenylserine	2.3 ± 2.4	−0.4 ± 1.1
L-Serine ethyl ester	3.7 ± 2.3	3.3 ± 3.9
D,L-Serine hydroxamate	0.0 ± 5.3	5.5 ± 4.3
L-Serine-*O*-sulfate	3.2 ± 2.1	11 ± 5
L-Serinamide	1.2 ± 4.1	−2.0 ± 3.9
D,L-Serinol	5.4 ± 0.8	−0.8 ± 3.0
Serine analogs		
β-Chloro-D,L-alanine	3.3 ± 3.2	4.6 ± 1.9
β-Cyano-L-alanine	19 ± 2	9.7 ± 1.6
D-Cycloserine	0.3 ± 0.9	−2.6 ± 2.3
L-Cycloserine	6.7 ± 5.3	−0.1 ± 4.4
Amino acid phosphonates		
D,L-AP4	19 ± 2 [a]	13 ± 3 [a]
D,L-AP5	5 ± 3 [a]	−7 ± 4 [a]
D,L-AP6	−1.1 ± 1.7	−6.4 ± 0.4
D,L-AP7	−0.4 ± 2.4	0.5 ± 3.0
Others		
Choline chloride	0.4 ± 1.3	−7.8 ± 4.6
Dithiothreitol	5.6 ± 3.6	3.5 ± 2.7
Iodoacetic acid	3.5 ± 2.2	0.1 ± 5.1

[a] Corrected for color formation in the absence of substrate, presumably due to contaminating inorganic phosphate. Compounds (1 mM in duplicate) were incubated with partially purified enzyme and 100 μM L-phosphoserine or 500 μM D-phosphoserine as substrate for 30 min at 37°C. Values are means ± S.E.M. of at least three independent experiments.

derivatized or modified α-carboxyl group (L-serine ethyl ester, D,L-serine hydroxamate, L-serinamide and D,L-serinol) were inactive. In addition, derivatization of the α-carbon (α-methyl-D,L-serine) or the β-carbon (D,L-*threo*-β-phenylserine) resulted in inactive compounds. The cyclic serine analogs (D- and L-cycloserine) were also inactive.

3.2. Effect of sulfhydryl reagents on phosphoserine phosphatase activity

CMPSA was a potent inhibitor of phosphoserine phosphatase activity using L-phosphoserine as substrate (IC_{50} 9.3 μM; Table 1, Fig. 4A) but had very low inhibitory activity using D-phosphoserine as substrate (IC_{50} 2900 μM; Table 1, Fig. 4B). In L-phosphoserine substrate saturation experiments, 20 μM CMPSA decreased the V_{max} of phosphoserine phosphatase with little change in K_m (Table 3, Fig. 5A) suggestive of noncompetitive inhibition, consistent with the Lineweaver–Burke transformation of the data (Fig. 5B).

Interestingly, *N*-ethylmaleimide inhibited phosphoserine phosphatase activity using L-phosphoserine as substrate (Table 1, Fig. 4A), but stimulated activity using D-phosphoserine as substrate (Table 1, Fig. 4B). The modulatory activity of *N*-ethylmaleimide was pH-dependent in

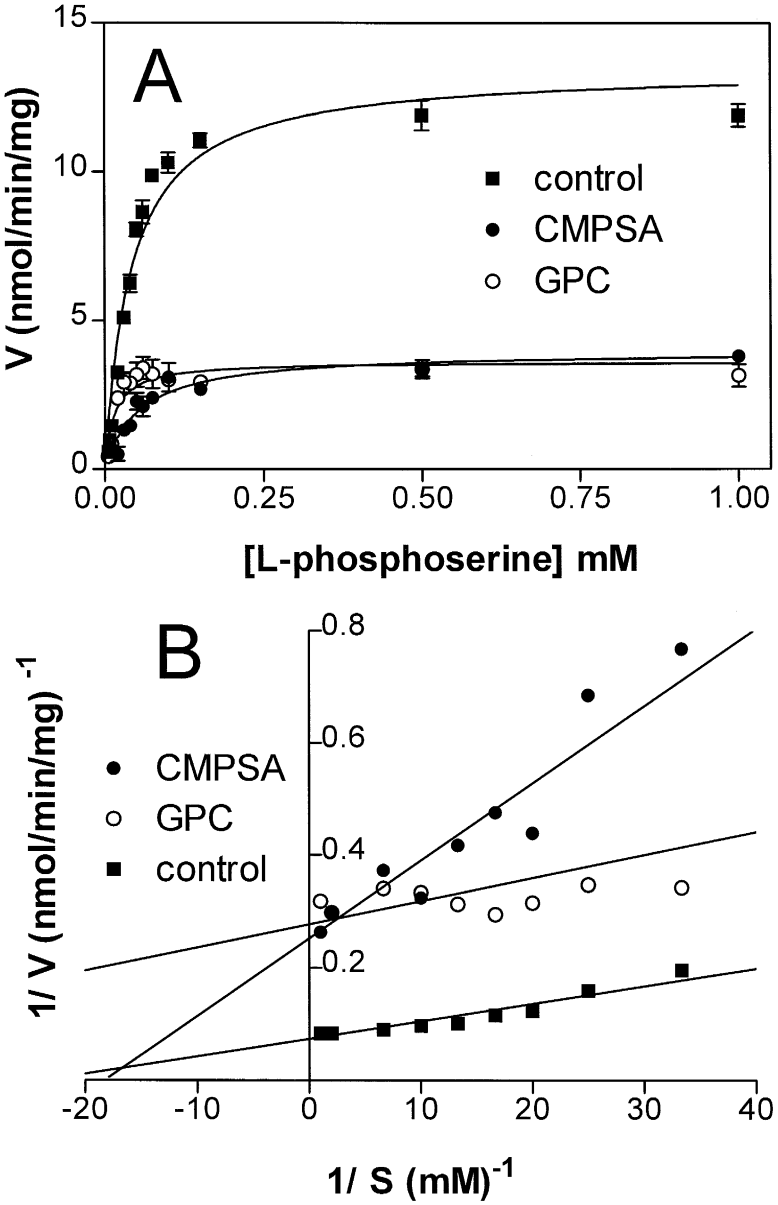

Fig. 5. Saturation of rat brain phosphoserine phosphatase in the absence and presence of *p*-chloromercuriphenylsulfonic acid (CMPSA) or L-α-glycerophosphorylcholine (GPC). Partially purified enzyme incubated with increasing concentrations of L-phosphoserine for 30 min at 37°C in the absence (control) or presence of 20 μM CMPSA or 15 μM glycerophosphorylcholine. (A) Hyperbolic plot. (B) Lineweaver–Burke transformation of the same data. The K_m and V_{max} values are shown in Table 3.

Table 3
Saturation of rat brain phosphoserine phosphatase in the presence of *p*-chloromercuriphenylsulfonic acid (CMPSA) and glycerophosphorylcholine

Inhibitor	K_m (μM)	V_{max} (nmol/min per mg)
Control	42 ± 3	13.5 ± 0.4
CMPSA	57 ± 10	4.0 ± 0.1
Glycerophosphorylcholine	15 ± 2	3.6 ± 0.2

Partially purified enzyme incubated with increasing concentrations of L-phosphoserine for 30 min at 37°C in the absence (control) or presence of 20 μM CMPSA or 15 μM glycerophosphorylcholine. Values are means ± S.E.M. of five (control) or three (CMPSA, glycerophosphorylcholine) independent experiments.

that *N*-ethylmaleimide was more potent at pH 7.5 than at pH 6.2, the standard assay condition chosen to provide optimal activity (Bridgers, 1967). Thus, *N*-ethylmaleimide was 10-fold more potent as an inhibitor of phosphoserine phosphatase activity using L-phosphoserine as substrate and was 24-fold more potent as a stimulator using D-phosphoserine as substrate at pH 7.5 as compared to pH 6.2, although the latter activity was difficult to quantify due to the low levels of product formation under these conditions. The sulfhydryl reagent iodoacetic acid and the disulfide reducing agent dithiothreitol were inactive (Table 2).

3.3. Characterization of glycerophosphorylcholine inhibition

In L-phosphoserine substrate saturation experiments, 15 μM glycerophosphorylcholine decreased the V_{max} of phosphoserine phosphatase and increased the apparent K_m (Table 3, Fig. 5A) suggestive of uncompetitive inhibition, consistent with the Lineweaver–Burke transformation of the data (Fig. 5B).

4. Discussion

Phosphoserine phosphatase is the rate-limiting enzyme in the L-serine synthetic pathway in brain. Since L-serine is metabolized to glycine via serine hydroxymethyl transferase, phosphoserine phosphatase may also be important for regulating central nervous system (CNS) glycine levels. Indeed, the glycine concentration in rat brain during development has been correlated with phosphoserine phosphatase activity (McChesney et al., 1987). Phosphoserine phosphatase may also be a primary regulator of brain D-serine levels either directly or indirectly (Fig. 1) (Wood et al., 1996). Whereas glycine is the endogenous agonist at strychnine-sensitive inhibitory glycine receptors, both D-serine and glycine probably function as co-agonists at the 'glycine' site of NMDA receptors in rostral and caudal CNS regions, respectively (Wood, 1995). Inhibitors of phosphoserine phosphatase may be useful in examining the contribution of endogenous D-serine and/or glycine to NMDA receptor function.

L-Serine was the first, and until recently, the only known micromolar phosphoserine phosphatase inhibitor identified (IC_{50} 500–600 μM; Hawkinson et al., 1996). L-Serine is a negative feedback inhibitor which regulates the metabolic activity of this enzyme. Allosteric inhibition by L-serine has been characterized as uncompetitive (Veeranna and Shetty, 1991). The binding pocket appears to be highly selective for L-serine since several types of analogs are inactive. Thus, analogs lacking the β-hydroxyl group or containing a derivatized β-hydroxyl group, a derivatized or modified α-carboxyl group, a derivatized α-amino group, or a derivatized α- or β-carbon are inactive. Similarly, L-serine analogs containing an elongated side chain, the hydroxy-amino regioisomer and the enantiomer (D-serine) are inactive, as are cyclic serine analogs.

The only known competitive inhibitor of phosphoserine phosphatase is the metabotropic glutamate receptor antagonist AP3, which is a structural analog of phosphoserine (Fig. 2) (Hawkinson et al., 1996). Unlike the stereoselectivity at the metabotropic receptor, the D-isomer of AP3 is a more potent phosphoserine phosphatase inhibitor. All other amino acid phosphonates examined are inactive, including AP4. Although AP4 appears to be a closer structural analog of the substrate phosphoserine than AP3 (Fig. 2), the surprising inactivity of AP4 may indicate that the active site does not accept molecules any longer than phosphoserine. Based upon crystal structures, the distance between the β-carbon and the phosphorus atom in AP4 (2.78 Å) (calculated from Chekhlov, 1992) is larger than in phosphoserine (2.41 Å) (calculated from Putkey and Sundaralingam, 1970). The corresponding distance in AP3 estimated by conformational analysis is considerably shorter (1.8 Å).

Several new phosphoserine phosphatase inhibitors related to phosphorylcholine are described in the present report, of which the glycerol ester glycerophosphorylcholine is the most potent (IC_{50} 18 and 31 μM using L- and D-phosphoserine as substrate, respectively). Glycerophosphorylcholine is a feedback inhibitor of lysophospholipase (Burt and Ribolow, 1994) and a metabolic precursor of phosphorylcholine (Yuan et al., 1992). Phosphorylcholine itself is 13- or 32-fold less potent than glycerophosphorylcholine as an inhibitor using L- or D-phosphoserine as substrate, respectively. Other esters of phosphorylcholine are active inhibitors, such as hexadecylphosphocholine, in which the phosphoryl group is esterified to 1-hexadecanol instead of glycerol. Hexadecylphosphocholine is equipotent with glycerophosphorylcholine using L-phosphoserine as substrate, but is 5.5-fold less active using D-phosphoserine as substrate. Other esters of phosphorylcholine are probably also phosphoserine phosphatase inhibitors. For these inhibitors, choline must be esterified to the phosphoryl group since choline chloride itself is inactive.

Although glycerophosphorylcholine has structural similarity to phosphoserine (Fig. 2), saturation experiments using L-phosphoserine as substrate indicated that the inhibition of phosphoserine phosphatase by glycerophosphorylcholine is uncompetitive rather than competitive. Uncompetitive inhibition is observed when the inhibitor binds only to the enzyme–substrate complex, resulting in a decrease in maximal velocity as well as an apparent increase in affinity due to a mass action shift to the right. This profile is also consistent with a mixed type inhibition in which the inhibitor produces a real increase in substrate affinity as well as a decrease in maximal velocity. In either case, the inhibition must be allosteric in nature. Glycerophosphorylcholine may bind either the L-serine feedback site or a novel site on the enzyme. The latter possibility is favored considering the exquisite selectivity of the feedback site for L-serine. Whole brain levels of glycerophosphorylcholine (0.4 μmol/g) (Ansell and Spanner, 1982) suggest that this water soluble intermediate in phospholipid metabolism may be a physiological regulator of phosphoserine phosphatase.

The effects of known inhibitors of phosphoserine phosphatase, sulfhydryl reagents and fluoride ion (Subrahmanyam, 1963), were more fully characterized. As suggested by previous studies (Subrahmanyam, 1963), phosphoserine phosphatase is inhibited by fluoride ion (IC_{50} 770 and 390 μM using L- and D-phosphoserine as substrate, respectively). Fluoride probably binds the active site and may block hydroxide ion reaction with the transition state by analogy to other Mg^{2+}-dependent enzymes, including ATPases (Murphy and Coll, 1992), enolase (Lebioda et al., 1993) and pyrophosphatase (Bayknov et al., 1992).

The effect of sulfhydryl reagents depends on the substrate used to measure phosphoserine phosphatase activity. Thus, the organic mercurial CMPSA is a potent inhibitor of the enzyme using L-phosphoserine as substrate (IC_{50} 9 μM) but is > 300-fold less potent using D-phosphoserine as substrate (IC_{50} 2900 μM). This finding accounts for the weak activity of CMPSA reported previously since D-phosphoserine was used as substrate (Subrahmanyam, 1963). Using L-phosphoserine as substrate, the sulfhydryl alkylator *N*-ethyl maleimide is 10-fold more potent as an inhibitor of phosphoserine phosphatase at pH 7.5 (IC_{50} 42 μM) than under the standard assay condition of pH 6.2. In contrast, *N*-ethylmaleimide enhances D-phosphoserine cleavage and the stimulatory effect is more potent at pH 7.5 than at pH 6.2. The enhancement of D-phosphoserine cleavage by *N*-ethylmaleimide occurs at similar concentrations as the inhibition of L-phosphoserine cleavage. The pH effect is consistent with the decreased reaction rate of *N*-ethylmaleimide at lower pH.

The stimulation of D-phosphoserine cleavage by *N*-ethylmaleimide and the noncompetitive inhibition of L-phosphoserine cleavage by CMPSA from saturation experiments indicate that the critical sulfhydryl residue(s) modified by these reagents occurs outside of the active site. Phosphoserine phosphatase is known to self-associate via intermolecular disulfide bonds under the conditions of the partial purification procedure (Paoli et al., 1974). Since CMPSA and *N*-ethylmaleimide are expected to dissociate these higher molecular weight forms, the potent inhibitory effect of these sulfhydryl reagents suggests that L-phosphoserine is preferentially cleaved by multimeric forms of the enzyme. Conversely, the weak inhibition by CMPSA and the stimulation by *N*-ethylmaleimide suggest that D-phosphoserine is preferentially cleaved by the monomeric form of the enzyme.

The current study expands the repertoire of known phosphoserine phosphatase inhibitors. The potent, noncompetitive inhibition by the organic mercurial CMPSA and the sulfhydryl alkylator *N*-ethylmaleimide using L-, but not D-, phosphoserine as substrate illustrates the importance of cysteine residues on the tertiary and possibly quaternary structure of this enzyme. The uncompetitive inhibitor glycerophosphorylcholine is considerably more potent than the previously known uncompetitive inhibitor L-serine and the recently reported competitive inhibitor AP3, particularly using the natural substrate L-phosphoserine. Stable analogs of glycerophosphorylcholine may provide useful tools in in vivo studies exploring the contribution of phosphoserine phosphatase to the endogenous pool of D-serine in brain.

5. Note added in proof

The recent report suggesting that D-serine is formed from L-serine by a racemase (Dunlop and Neidle, 1997) requires demonstration of L-serine to D-serine epimerization by the pure mammalian enzyme. Human phosphatase has recently been cloned and expressed (Collet et al., 1997)

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