Serine racemase (SR) is a brain enzyme present in glial cells, where it isomerizes L-serine into D-serine that, in turn, diffuses and co-activates the N-methyl-D-aspartate receptor through the binding to the so-called glycine site. We have developed a method for the slow expression of SR in a eukaryotic vector that permits the correct insertion of the prostatic group into the active site, rendering functional SR with a K_m toward L-serine of 4.8 mM. Divalent cations such as calcium or magnesium were necessary for complete enzyme activity, whereas the presence of chelators such as EDTA completely inhibited the enzyme. Moreover, direct binding of calcium to SR was evidenced using 45Ca^{2+}. Gel filtration of the recombinant SR revealed the protein to be in a dimer-tetramer equilibrium. The addition of EDTA to a calcium-saturated serine racemase evokes a profound conformational change, as monitored by both fluorescence and circular dichroism techniques. Fluorescence titration allowed us to calculate a binding constant for calcium of 6.2 μM. Reagents that react with sulfhydryl groups, such as cystamine, were potent inhibitors of SR, in a clear reflection that one or more cysteine residues are important for enzyme activity. Additionally, 16 serine analogues were tested as a putative SR substrate or inhibitors. Significant inhibition was only observed for L-Ser-O-sulfate, L-cycloserine, and L-cysteine. Finally, activation of brain SR as a result of the changes in calcium concentration was studied in primary astrocytes. Treatment of astrocytes with the calcium ionophore A23187, as well as with compounds that augment the intracellular calcium levels such as glutamate or kainate led to an increase in the amount of D-serine present in the extracellular medium. These results suggest that there might be a glutamatergic-mediated regulation of SR activity by intracellular calcium concentration.

D-Amino acids have been known for decades to be present in bacteria, where they are important constituents of peptidoglycan in the cell wall. Interestingly, recent improvement in the detection techniques has allowed the identification of significant levels of both D-serine (1–3) and D-aspartic acid (3, 4) in the nervous system of vertebrates. Snyder and co-workers (5, 6) have elegantly purified and subsequently cloned the cDNA for a novel enzyme responsible for the synthesis of D-serine in the brain and identified it as a 37-kDa pyridoxal phosphate-containing racemase present in astrocytes. These protoplasmic astrocytes typically ensheathe synapses, strongly suggesting a role for D-serine in synaptic transmission. Serine racemase is highly enriched in the brain and co-localizes with D-serine according to immunohistochemical analysis (6). Additionally, D-serine is concentrated in regions enriched in N-methyl-D-aspartate (NMDA)^1 receptors (i.e., highest in the forebrain), whereas the levels of the previously identified NMDA receptor coactivator, glycine, are lowest in this region (7, 8). Remarkably, recent reports have also identified D-serine in neuronal cells, such as a pyramidal neurons in the cerebral cortex and neurons in the nucleus of the trapezoid body (9).

The initial observation of Kleckner and Dingledine (10) that D-serine could substitute for glycine in terms of NMDA receptor coactivation when transfected in Xenopus oocytes was followed by its confirmation in cultured neurons (11, 12). NMDA receptor channel activity can be potentiated by concentrations as low as 6.5–10.0 μM D-serine (13, 14). On the other hand, support for the role of D-serine as a neuromodulator is strengthened by the fact that both glutamate and agonists of non-NMDA glutamate receptors, such as kainate, promote the release of D-serine from astrocytes (7). Additionally, selective degradation of extracellular D-serine using exogenously added amino acid oxidase greatly attenuates NMDA receptor-mediated neurotransmission, as monitored by the increase in neuronal nitric-oxide synthase activity or cGMP levels (15).

Inspection of the murine (6) and human (16) serine racemase cDNA reveals an overall sequence identity of 88%. In addition, putative homologs of serine racemase with previously unknown function are also found in Saccharomyces cerevisiae, Caenorhabditis elegans, and Arabidopsis thaliana (6). The PLP-binding region of serine racemase is located in the N-terminal region of the protein and displays significant homology to other members of the PLP family, such as the enzymes of the serine/threonine dehydratase family (6, 16). Recently, recombinant mouse serine racemase isolated from mammalian cells fused to glutathione S-transferase has been used to show that L-serine O-sulfate is a nonnatural substrate and inhibitor, which is able to transform its racemase activity into that of an eliminase (17). Since the eliminase reaction toward the alternative substrate is 500 times faster than its natural reaction, detection of the presence of serine racemase in cultures can become greatly improved (17).
We report herein the characterization of recombinant brain serine racemase expressed in *E. coli* in large quantities. The recombinant enzyme was active only in the presence of divergent cations such as calcium and manganese. The binding of calcium to the enzyme results in major conformational changes. Finally, we demonstrate that both glutamate- and A23187 ionophore-induced calcium entrance in astrocytes also lead to the activation of the serine racemase with a concomitant increase in the detected extracellular concentration of D-Ser.

MATERIALS AND METHODS

**Chemicals**—Ultrapure l-serine was purchased from NovaBiochem (Switzerland). d-Serine, l-cysteine, guanidinoacetic acid, O-methyl-l-serine, cystamine dihydrochloride, β-chloro-l-alanine, and amino acid oxidase from porcine kidney were from Sigma. l-serine, l-serine-O-benzyl, l-serine methyl ester, l-serine-O-sulfate, and l-serine-O-acetate were from Bachem (Switzerland). R(−)-1-aminoethyolphosphonic acid, n-cycloserine, and L-2-aminobutyric acid were from Fluka. l-Cycloserine, l-homoserine, benzoylacetethyldehydide acetyl, and acetaldehyde were from Aldrich. l-Threonine and phosphorous trichloride were from Merck. Horseradish peroxidase was from Roche Molecular Biochemicals. 4°C CaCl2 was purchased from Amersham Pharmacia Biotech. Anti-serine racemase antibodies were purchased from Transduction Laboratories.

**Construction of the pCWori-SR Plasmid and Recombinant Protein Expression**—The plasmid encoding the cDNA of mouse serine racemase was a generous gift from Dr. S. H. Snyder (Department of Neuroscience, Johns Hopkins University). Two oligonucleotides were used to amplify the cDNA: 5'-AGGCAAGCAGACATTATGGT-3' and 5'-TCTCTGCTGATTTAATGAGGAA-3' were novel NdeI and XbaI sites were introduced at the initiation Met residue and at the stop codons, respectively (underlined sequences). The amplified fragment of DNA (~1,030 bp) was subcloned into a pGEM-T vector (Promega) and confirmed by automated DNA sequencing. The pGEM-T-SR plasmid was double-digested with NdeI plus XbaI, and the excised fragment of DNA was inserted into the corresponding site of the poly-His-pCWori vector (18). BL21 (DE3)-competent cells (Novagen) were transformed with the pCWori-SR vector and an overnight 50-ml culture was used to inoculate each of three 2-liter flasks containing 1.5 liters of 2YT medium.

**Purification of Recombinant Serine Racemase**—The frozen cell paste was resuspended in buffer A (20 mM Mops, pH 8.0, 50 mM NaCl, with 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM pepstatin) plus 0.5 mM mg/ml ampicillin. The cultures were grown at 37 °C to an absorbance of 2.0 and induced by the addition of 2 mM isopropyl-thio-β-D-galactopyranoside. The four flasks of bacterial cultures were harvested at 30 °C at 240 rpm for 16–20 h before the cells were harvested by centrifugation and frozen in plastic bags as thin films and stored at −80 °C. Construction of a recombinant SR with a removable tag was performed using the oligonucleotide 5'-ATTAATCTGTTTCCGCCTG- GATTCCGTCAGTACGTG-3' that includes a novel Val-Pro-Arg-Gly-Phe-Ala sequence that can be cleaved by thrombin. This oligonucleotide was used to amplify the 5' end of the cDNA.

**Purification of Recombinant Serine Racemase**—The frozen cell paste was resuspended in buffer A (20 mM Mops, pH 8.0, 50 mM NaCl, with 0.5 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, and 1 mM pepstatin) plus 0.5 mM mg/ml ampicillin. The cells were lysed by sonication (three cycles of 5 min at medium potency on ice) and centrifuged in a SS34 rotor (Sorvall) for 30 min at 14,000 rpm. All the purification steps were performed at 4 °C. Since the pCWori used to express the recombinant SR has a hexahistidine tag translated in frame at the amino end of the coding region (18), purification of the protein was initially performed by affinity chromatography. The clear cellular supernatant was then passed through Whatman filter paper before loading on a 5-ml Ni2+-nitrilotriacetic acid column (Qia gen). The column was washed with 25 ml of buffer A followed by another 25 ml of buffer A plus 300 mM imidazole. The protein was subsequently eluted with buffer A plus 200 mM imidazole. The yellow fractions were collected, 0.5 mM pyridoxal phosphate was added, and they were extensively dialyzed against 100 mM ammonium carbonate, pH 8.5, 5 mM NaCl. After dialysis, the samples were lyophilized and then resuspended in 1 ml of 20 mM Mops, pH 8.0, 50 mM NaCl, 0.5 mM DTT and loaded onto a Superdex 200 HR column equilibrated in the same buffer. Approximately 200 μl were injected each time, and the column was run at 1 ml/min. The fractions eluting at ~11–13 ml, which corresponded to both dimers and tetramers of SR (see below), were collected and pooled. We discarded fractions eluting at larger volumes due to the contamination with smaller fragments. The pooled fractions were frozen in liquid nitrogen and kept at −20 °C. When the removal of the recombinant tag by thrombin was required, this additional step was introduced between the affinity column and the gel filtration column in order to remove any traces of thrombin that might be present in the latter purification step. Identical elution profiles were obtained in the Superdex 200 HR column in the absence or presence of the His tag.

**Circular Dichroism Measurements**—CD spectra were recorded on a Jasco J-715 spectropolarimeter using either a 0.1-cm path length cell for the far UV measurements or a 1-cm path length cell for the near UV and visible measurements, in all cases at 25 °C. The temperature in the cuvette was regulated with a Neslab RT-11 circulating water bath. The protein concentration was 0.1–0.5 mg/ml. The buffer used was 50 mM ammonium carbonate, pH 7.8, 10 mM NaCl, 0.5 mM DTT. A minimum of five spectra were accumulated for each sample, and the contribution of the buffer was always subtracted. The resultant spectra were smoothed using J715 noise reduction software. The values of mean residue molar ellipticities [θ] (degrees × cm2 × dmol−1) were calculated on the basis of an average Mr value of 110 per residue. Deconvolution of the circular dichroism data was performed according to the convex constraint analysis method (19), which relies on an algorithm that calculates the contribution of the secondary structure elements that give rise to the original spectral curve without referring to spectra from model systems.

**Thermal denaturing curves** of recombinant SR of either 1 mM calcium or 1 mM EDTA were recorded at 1 °C/min using a 1-cm cellulose filter of pure SR Tris, 20 mM Mops, pH 7.8, 20 mM NaCl, 1 mM DTT. Ellipticity in millidegrees was measured at 220 nm. The temperature of the CD cuvette was regulated by a Neslab RT-11 water bath that was computer-controlled.

**Fluorescence Measurements**—Fluorescence studies were carried out on a SLM Amino 8000C spectrofluorimeter, fit with a 450-W xenon arc, using 4-nm slits for both excitation and emission beams. A 0.4-cm excitation path length and a 1-cm emission path length quartz cell was used. In all cases, the protein concentration was 0.05–0.1 mg/ml in 20 mM Mops, pH 7.8, 50 mM NaCl buffer. The temperature in the cuvette, 25 °C, was maintained by a circulating water bath. Excitation was performed at 275 or 285 nm, and emission spectra were measured over a range of 280–450 nm. The contribution of the background (buffer) was subtracted in every case, and the spectra were then corrected for the instrument response according to the program software. The binding constant for calcium was obtained from the x intercept of a double reciprocal plot of (F−F0)/(F−F0) versus the calcium concentration, where F is the fluorescence signal 320 nm at a certain calcium concentration, F0 is the fluorescence signal at 320 nm in the presence of saturating concentrations of calcium, and F0 is the fluorescence signal in the absence of calcium.

**Gel Filtration Analysis of Recombinant Serine Racemase**—Aliquots of 100 μl of ~2 mg/ml of recombinant racemase were injected into a GP 250 plus fast protein liquid chromatography system equipped with two P-500 pumps and a Superdex HR200 column (Amersham Biosciences). Separation was performed at 25 °C, and protein detection was performed at 280 nm. The flow rate was kept at 1 ml/min, and the buffer included 50 mM NaCl, 1 mM DTT, 20 mM Mops, pH 7.8, 10 mM NaCl. The column void volume was determined with dextran blue, and the total volume was determined with potassium ferricyanide. The column was calibrated with proteins from a gel filtration kit (Sigma), including thyroglobulin (669 kDa), apoferritin (443 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and myoglobin (17 kDa). The calibration curve was obtained by plotting the (vV−vV0)/vV0 ratio against the log of the molecular mass, where vV is the elution volume of the protein, vV0 is the void volume of the column, and vV0 is the total bed volume of the column.

**Enzyme Assays**—A routine colorimetric assay with 200 μl of total sample was used with l-serine as a substrate, coupling the appearance of d-serine to commercial l-α-amino acid oxidase and horseradish peroxidase, plus the peroxidase substrate o-phenylenediamine. The l-serine that was produced during the incubation period was degraded by l-α-amino acid oxidase, which specifically targets l-α-amino acids generating α-keto acid, ammonia, and hydrogen peroxide. The hydrogen peroxide was quantified using horseradish peroxidase and o-phenylenediamine, which turns yellow upon oxidation. The activity of serine racemase was determined in the presence of 20 mM Mops, pH 8.1, 0.5 mM phenylalanine, 3 mg/ml of purified l-Ser, 0.93 mM DTT, 5 μM PLP, 50 μg/ml o-phenylenediamine, 1 mM FAD, 0.2 mg/ml l-α-amino acid oxidase, and 0.01 mg/ml horseradish peroxidase. The reactions (200-μl final volume) were incubated at 37 °C for 2 h before measuring the absorbance at 411 nm with a Beckman DU-7 spectrophotometer. The l-serine present in a given sample was determined by correlating the absorbance at 411 nm with l-serine calibration curves. d-Serine measure-
ments were not influenced by the concentration of L-isomers present in the sample. Since standard commercial L-serine preparations contain trace amounts of D-serine, it was necessary to purchase ultrapure L-serine from Bachem Inc. (Switzerland) to achieve a reasonable signal-to-noise ratio. Once optimized, this three-enzyme assay was able to detect D-serine from an unknown 100-μl sample in a linear range from 50 μM to 1 mM (up to 0.1 μM total of D-serine). Since it is possible that certain serine analogues are capable of being substrates for L-amino acid oxidase (causing an artificial increase in signal), as well as potential inhibitors of D-amino acid oxidase or horseradish peroxidase (causing an artificial decrease in signal), appropriate controls were in place to identify and, subsequently, account for these effects.

Inhibition curves were fitted to a sigmoidal curve using the equation,

\[
y = a/(1 + \exp(-(x-x_0)/b))
\]

(Eq. 1)

where \(y\) represents the percentage SR activity, and \(x\) is the inhibitor concentration. Every inhibition curve was systematically repeated a minimum of three times, with the error bars indicating one S.D.

Additionally, we used an HPLC assay when the concentrations of D-serine to be determined were below the 100 μM range. Derivatization of D-serine and L-serine mixtures was performed with Marfey reagent (1-bromo-2,4-dinitrophenyl-5-L-alanine amide) (Fierce) as previously described (20). In summary, 50 μl of the problem sample were mixed with 20 μl of a 1.0 M solution of sodium bicarbonate. Then 200 μl of Marfey reagent (1% solution in acetone) were added, and the mixture was incubated at 40 °C for 1 h in a block heater. The mixture was subsequently cooled down, and 10 μl of 2 m HCl were added. Separation of the D-serine from L-serine was performed using a C-18 Kromasil 100 column (15.0 × 0.46 cm) isocratically using methanol as the eluent in a 0.02 M sodium acetate buffer (pH 4.0) plus methanol (80:20) (21). The absorbance was monitored at 340 nm with a Beckman detector module 166. Calibration curves were constructed after injection of known amounts of L- and D-serine.

**Synthesis of the Phosphonic Acid Analogue of Serine—**L-Amino-2-hydroxyethylphosphonic acid was synthesized according to the method described by Lejczak and co-workers (22) with slight modifications. Acetamide (1.18 g, 20 mmol) was mixed with glacial acetic acid (3 ml) before cooling to 0 °C. To the stirred solution was added acetyl chloride (710 μl, 10 mmol) dropwise over a period of 5 min, causing the formation of a white precipitate. Benzoylxyacetaldehyde diethyl acetal (2.02 ml, 10.0 mmol) was added, and the reaction was stirred at room temperature overnight. Phosphorous trichloride (970 μl, 10 mmol) was added to the red reaction before refluxing for 1.5 h. The solution was cooled and concentrated under reduced pressure. The dark syrup was dissolved in concentrated HCl (30 ml), refluxed for 7 h, and evaporated using a rotary evaporator. The resultant residue was taken up in water (50 ml) and treated with activated charcoal before concentrating to a few ml. The solution then passed through a Dowex 50W X8 (acetate form), eluting with water. The nihinydrine-positive fractions were combined and concentrated to <0.5 ml, and the product was subsequently precipitated with absolute ethanol. Filtering yielded ~50 mg of pure product as confirmed by the proton NMR and mass spectra.

**Binding of \(^{44}\text{Ca}^2+\) to Reombinant SR—**We tested the binding of radioactive calcium to serine racemase both in dot-blot and in a sample that was transferred to a nitrocellulose membrane after SDS-PAGE. In either case, the nitrocellulose membrane containing 0.5 mmol of SR (SDS-PAGE sample) or 2 mmol of SR (dot-blot sample) was washed for 5 min with 2 ml EGTA followed by three 5-min washes with 10 mM Pipes, pH 6.9, 50 mM NaCl, 0.1 mM MgCl\(_2\). Afterward, the membranes were incubated for 15 min with 6 μM \(^{44}\text{CaCl}_2\) (5,000 mCi/mmol) in the same buffer. The excess of \(^{44}\text{CaCl}_2\) was removed with two 30-s washes in 500 ml of distilled water. The membranes were subsequently allowed to dry, and they were used to expose photographic film for 8–12 h at ~80 °C.

**Cell Culture—**Cortical astrocytes were derived from 24-h Wistar rats and cultured in serum-containing medium as described before (23). For all of the experiments, determinations performed, the serum-containing medium was removed, and cells were transferred to a chemically defined, serum-free medium consisting of BME basal medium supplemented with 5 μg/ml insulin, 50 μg/ml transferrin, 20 ng progesterone, 50 μM putrescine, 30 mM sodium selenite, and 10 mM L-Ser of the highest purity available. The absence of Ser in the commercial media was strictly necessary in order to avoid high levels of contaminating D-Ser.

**RESULTS**

Purification and Enzymatic Characterization of Reombinant Serine Racemase—pCWori is known to be a suitable vector for the recombinant expression of proteins in E. coli with prosthetic groups bound. Unlike vectors with strong promoters, such as the T7 promoter of pET plasmids, recombinant expression of proteins in pCWori leads to a steady and constant synthesis of the desired polypeptide that typically allows the E. coli machinery to keep up with other cellular functions, such as the formation and insertion of protein cofactors. In the present work, cloning of the SR gene into pCWori, transformation of BL21 E. coli cells with pCWori-SR, and growth of cells at 30 °C resulted in expression of functional SR as determined by activity assays. Expression of the protein with the polyhistidine tag yielded 3–6 mg of purified protein from 6 liters of culture. Purification using the Ni\(^{2+}\)-nitrilotriacetic acid-agarose affinity column provided protein that was ~75% pure as judged by SDS-PAGE (Fig. 1A). Improvement of the purity of the serine racemase preparations was obtained after lyophilization of the dialyzed sample (in order to remove the imidazole) followed by the loading of the protein onto a gel filtration column. This second purification step yielded an homogenous protein preparation as judged by SDS-PAGE (Fig. 1A). The faint larger bands that could be observed in the gel reflected the presence of dimers and tetramers of the protein that resist the denaturing conditions, since they reacted with anti-SR antibodies. In this regard, aging of the enzyme led to the progressive increase in the relative ratio of these bands.

We developed a colorimetric activity assay for murine serine racemase based on the transformation of L-serine into D-serine, which was then used as a substrate of D-amino acid oxidase with the concomitant release of hydrogen peroxide. Detection of \(\text{H}_2\text{O}_2\) was performed with peroxidase and \(\alpha\)-phenylenediamine as substrate, which displays a defined yellow absorbance at 411 nm when it becomes oxidized. Analysis of the purified serine racemase activity using Michaelis-Menten kinetics rendered a \(K_m\) value of 4.8 mm when L-serine was used as a substrate (Fig. 1B). The \(V_{\text{max}}\) of SR, as determined by this colorimetric assay, was ~112 nmol·mg\(^{-1}\)·min\(^{-1}\) at 37 °C. These results agree with previous data reported for tissue-purified and recombinant protein obtained from mammalian cells (5, 17).

**Effect of Divalent Cations, NO-releasing Agents, and SH-modifying Compounds on SR Activity—**Incubation of recombinant SR with EDTA, a chelator of divalent cations, almost abrogates the synthesis of D-serine (Fig. 2A), whereas the incubation of the purified enzyme with both calcium and manganese results in an increase in the activity levels to 134 and 153%, respectively. This increase in the SR activity levels was also studied in the presence of other divalent cations; \(\text{Fe}^{2+}\) and \(\text{Ni}^{2+}\) had a very limited effect, whereas \(\text{Mg}^{2+}\) was capable of augmenting the activity to 112%. The addition of \(\text{Zn}^{2+}\) to the activity assay, on the other hand, led to lower activity levels (Fig. 2A). The existence of racemase activity in the recombinant enzyme in the absence of any added divalent cation very likely reflects a certain degree of saturation with cations that are strictly necessary in order to avoid high levels of contaminating D-Ser.

Since SR is known to be sensitive to the presence of oxidized glutathione (5) (probably due to the oxidation of one or more free sulfhydryls), we investigated whether nitric oxide might regulate the enzyme activity. The addition of DETA NONOate, a NO-releasing compound, to the enzyme mixture and incubation for up to 4 h did not significantly change the amount of D-serine synthesized (Fig. 2A). Analysis of S-nitrosylated serine racemase in vitro, performed using published methodologies
(24) with cytochrome c as a positive control, failed to detect the formation of nitrosylated cysteine residues in SR (data not shown).

Since divalent cations might interact with the hexa-His tag of the enzyme, we also constructed a vector with a cleavable tag at the N terminus end of the protein as reported under "Materials and Methods." Treatment with thrombin was performed after elution from the affinity column and prior to the Sephadex HR200 gel filtration column. This recombinant protein behaved identically to the protein that possessed the hexa-His tag in terms of stability, activation by calcium, or activity.

Next, we investigated the effect of increasing concentrations of calcium on the SR activity using the colorimetric assay. The enzyme was purified in the absence of any added cation, and it
was then incubated with 1 mM EDTA. Subsequently, increasing concentrations of Ca^{2+} were added, and the amount of D-Ser synthesized was determined. By means of this methodology, we were able to determine an EC_{50} for SR of about 26 μM (Fig. 2B).

However, the turnover of L-Ser into D-Ser by the racemase was severely affected by the addition of cystamine, a cysteine-blocking reagent, displaying an IC_{50} of 8.1 μM (Fig. 2C). These results suggest that the inactivation of the enzyme by both oxidized glutathione and cystamine might be due to either the oxidation or the blockade of a cysteine residue very likely involved in catalysis. Significantly, successful protein expression was achieved in a prokaryotic system, despite the different redox environment of E. coli when compared with the cytosol of the astrocytes.

**Inhibition by L-Serine Anallogues**—One of the most potent inhibitors of the PLP-containing homologous alanine racemase is the alanine phosphate (1-aminoethane phosphonic acid) (25, 26). Consequently, we synthesized 1-amino-2-hydroxyethane phosphonic acid (i.e. serine phosphonate), and together with the commercially available alanine phosphate tested them as putative inhibitors of the recombinant SR. The synthesis of the compound was performed as described under “Materials and Methods.” Additionally, 14 other serine analogues were also tested as putative inhibitors of brain SR: D,L-Ser methyl ester, L-homoserine, guanidineacetic acid, L-amino- butyric acid, L-threonine, O-benzyl-L-serine, O-methyl-L-serine, O-phospho-DL-serine, O-acetyl-L-serine, alanine phosphonic acid, serine phosphonic acid, β-chloroalanine, O-sulfate-L-serine, L-cycloserine, and L-cysteine (Fig. 3). In the presence of 10 mM L-serine and at a constant inhibitor concentration of 2 mM, the strongest inhibition was observed for L-cysteine, which virtually abrogated the racemase activity, followed by L-cycloserine and L-serine O-sulfate, with 38 and 57% activity, respectively. Surprisingly, the synthetic compound 1-amino-2-hydroxyethane phosphonic acid, the phosphonic acid analogue of serine, as well as 1-aminoethane phosphonic acid only induced a modest inhibition of the racemase activity. The only other compounds that showed some inhibition were L-Ser-O-acetate, D-cycloserine, and β-chloroalanine (−70 to −80% activity), although the degree of inhibition was rather limited (Fig. 3).

**Inhibition by L-Cysteine and L-Cycloserine**—Since two of the compounds that displayed a better inhibition of recombinant SR were L-cysteine and L-cycloserine, we decided to analyze both of them in a broad concentration range (Fig. 4). Although L-cysteine is structurally very similar to L-serine, the presence of a reactive sulfhydryl might lead to mixed effects, especially if disulfides were formed with the side chains of other residues in the protein, as suspected from the cystamine inhibition experiments (Fig. 2B). L-Cysteine inhibited SR with an IC_{50} of 0.25 mM (Fig. 4A). However, since L-cysteine is also known to react with some pyridoxal phosphate enzymes (27), we inspected the spectral changes that occur in the visible region upon the addition of L-cysteine. As observed in Fig. 4B, the addition of increasing concentrations of L-cysteine to SR caused the disappearance of the 415-nm peak with the concomitant appearance of a new peak at 330 nm, indicative that the inhibitor reacts with the PLP cofactor (see below). Inhibition of SR by L-cycloserine proceeds with an IC_{50} of 2.27 mM (Fig. 4C) and, apparently, is also due to the formation of covalent complexes between the inhibitor and PLP (Fig. 4D). According to the spectral changes observed, the addition of L-cycloserine led to the progressive inactivation of the enzyme.

**Spectroscopic Properties of SR**—The absorbance spectrum of recombinant SR in the 250–550-nm range showed a clear contribution of the PLP moiety in addition to that of the aromatic amino acids (Fig. 5A). The peak maximum of the PLP was centered at −415 nm (Fig. 5A, inset), and the ratio A_{280}/A_{415} for the fully saturated enzyme was 7.1. The absorbance spectrum of SR was not significantly changed by the presence of either calcium or EDTA (data not shown). When recombinant brain serine racemase was analyzed using circular dichroism, a negative contribution of the aromatic amino acids side chains in the 250–300-nm region followed by a positive contribution due to the PLP group was clearly observed (Fig. 5B). This important contribution of the pyridoxal phosphate group in the visible region was most likely due to a significant asymmetric environment in the immediacy of the cofactor.

The secondary structure of recombinant serine racemase was also analyzed using circular dichroism in the far UV range in the presence and absence of Ca^{2+} (Fig. 6A). The changes in the circular dichroism spectrum of SR when EDTA was added (open circles) compared with a calcium-saturated sample (open squares) were very small. These results lead to the conclusion that calcium binding does not lead to large conformational changes affecting the secondary structure of the protein. Deconvolution of the calcium-saturated CD spectra into its pure components was performed using the convex constraint algorithm (19) that relies on a matrix of multiple experimental data from proteins in solution. Accordingly, recombinant SR rendered 36.2% α-helix, 17.1% β-stands, 32.2% unordered structures, and 17.1% β-turns. The protein of known three-dimensional structure with the largest sequence homology to serine racemase is the biosynthetic E. coli threonine deaminase, essentially its N terminus domain (residues 1–320) (data not shown). The secondary structure prediction obtained for SR is in good agreement with the crystallographic data of the pyridoxal phosphate-containing threonine deaminase N-terminal domain, which consists of two subdomains with similar folds (a central four-stranded, parallel β sheet with multiple flanking α-helices and large loops connecting both elements) (28).

Significant changes were observed in the fluorescence spectra of SR in the presence of calcium and EDTA (Fig. 6, B and C). In the presence of 1 mM Ca^{2+}, the emission fluorescence spectrum of SR was studied after excitation at 275 nm, and the maximum of the spectrum was centered at 320 nm (Fig. 6B). Deconvolution of the spectrum into its components showed that most of the fluorescence emission was due to the contribution of the tryptophan residues (Fig. 6B, dotted curve), with a minor contribution of the tyrosine residues (Fig. 6B, dashed curve), as it usually occurs with most of the proteins (29). Interestingly, in the presence of EDTA, a large increase was observed in the quantum yield of the spectrum (Fig. 6C) as well as a red shift of the emission maximum to 326 nm. This enhancement in the fluorescence emission signal of SR was probably due to the contribution of the tryptophan residues. Thus, one or several tryptophan side chains that were partially quenched in the protein structure in the presence of calcium became exposed when EDTA was added. These results not only indicate that a prominent change in the proximities of the side chains of some aromatic residues occurs when calcium was bound but also provide direct evidence that the tight regulation of the catalytic activity of the enzyme in the presence or absence of calcium reported above correlates with changes in the tertiary structure of the serine racemase. These fluorescence changes from the calcium-saturated to the calcium-free form were highly reproducible and allowed us to determine a calcium binding constant for SR of 6.2 ± 1.2 μM.

**Thermal Denaturation of SR in the Presence and Absence of Ca^{2+}**—The intriguing possibility that a PLP enzyme is regulated by the calcium concentrations due to the direct binding of...
the divalent cation was explored using circular dichroism measurements of the enzyme at increasing temperatures (Fig. 7A). In the presence of 1 mM Ca$^{2+}$, a sharp transition was observed with a midpoint at $-56.5^\circ$C. This would agree with a two-state transition from a native to a denatured conformation of the protein. However, in the presence of the metal chelator EDTA, the sharp transition was lost, and a more complex process seemed to take place (Fig. 7A). In the absence of Ca$^{2+}$, the thermal denaturation of the protein would occur in at least two steps. The first one would be a sharp, but small transition, centered at about 35 °C followed by a second, less cooperative transition. This behavior would not fit within a two-state thermal transition, and its most likely interpretation would be a change in the oligomeric state of the protein corresponding to the transition at lower temperatures, followed by a much more complex unfolding of the protein. Consequently, these structural studies also provide direct evidence for the conformational changes arising upon calcium binding to the protein.
The direct binding of calcium to serine racemase was further confirmed by incubation of a nitrocellulose membrane containing the transferred recombinant enzyme with $^{45}\text{Ca}^{2+}$ (Fig. 7B). A clear band at the correct molecular mass was observed, whereas none of the molecular weight markers showed any calcium binding.

**Gel Filtration Chromatography of SR**—To study the oligomeric structure of recombinant SR we used a Superdex HR200 column, which resolves proteins approximately in the 30–900-kDa molecular mass range. The elution profile of serine racemase is depicted in Fig. 8A. A clear, sharp peak was apparent at 12.7 ml after injection, together with another peak corresponding to larger molecular masses eluting at about 11.4 ml. Calibration of this column using fast protein liquid chromatography protein standards and interpolation of the elution volume of SR within the calibration line gave us a theoretical molecular mass for serine racemase of 82.9 kDa (Fig. 8B). A dimeric structure can be inferred from this result, since each of the monomers of SR has a real mass of ~37.5 kDa (Fig. 8B). Interpolation of the peak eluting at 11.4 ml within the calibration line gave us a value of 153 kDa, most likely due to the tetrameric form of the enzyme. It is noteworthy that monomeric SR was completely absent, which leads to the conclusion that serine racemase exists in solution in a dimer-tetramer equilibrium clearly displaced toward the dimeric species. The presence of EDTA or calcium did not significantly change the elution profile of recombinant SR.

**Activation of Serine Racemase within Primary Astrocytes**—Finally, we investigated whether the addition of glutamate, kainate, and the calcium ionophore A23187 to astrocytes could result in the intracellular activation of glial serine racemase, resulting in the extracellular secretion of d-Ser. We collected the cell medium of astrocytes incubated with 300 μM glutamate, 100 μM kainate, or 500 nm calcium ionophore A23187 for 24 h and determined the amount of d-Ser by HPLC after reaction with Marfey reagent (Fig. 9A). Both glutamate and kainate markedly augmented the release of d-Ser from astrocytes when compared with the nonstimulated cells, reaching levels of 23 and 16 μM, respectively. Moreover, incubation of primary astrocytes with A23187 resulted in the synthesis of 21 μM d-Ser (Fig. 9), a result endowed with special relevance, since the activity of the recombinant SR was shown to be calcium-dependent. Similar levels of d-serine synthesis were observed at shorter times (8 and 12 h). Therefore, glutamatergic stimulation very likely results in increased intracellular calcium concentration that activates serine racemase and ultimately raises the levels of extracellular d-Ser. Finally, in order to rule out an augmentation in the serine racemase expression levels due to the treatment with glutamate, kainate, or A23187, we performed an immunodetection of the SR levels present within...
astrocytes in every case using anti-SR antibodies (Fig. 9B). As depicted in the figure, none of the compounds resulted in a significant increase in the protein levels of the enzyme.

**DISCUSSION**

Identification of D-serine as a novel yet atypical neurotransmitter has only been accepted as such in the past few years (30). Evidence for the role of astrocyte-derived D-serine as a neurotransmitter includes (i) its release following glutamatergic activation of non-NMDA receptors (7), (ii) the depression observed in NMDA transmission following selective degradation of D-serine by exogenous d-amino acid oxidase (15), (iii) the ability of D-serine to fully mimic the activity of the endogenous transmitter at the glycine site of the receptor (10, 13–15), (iv) the specific localization of the synthesized D-serine in synapses in close proximity to the NMDA receptors (7), and (v) the selective localization of serine racemase in astrocytes (5–7).

Physiological activation of the NMDA receptors requires the concerted action of glutamate and D-serine. However, the overproduction of glutamate has been implicated in a large number of both acute and chronic degenerative conditions, including epilepsy; stroke; peripheral neuropathies; Alzheimer’s, Huntington’s, and Parkinson’s disease; and chronic pain (see Refs. 31–35 and references therein). During all of these pathological challenges, excessive glutamate is released, which is highly toxic to neurons. The harmful effects of excessive glutamate are believed to occur principally through activation of the NMDA receptor. On the other hand, D-serine is known to be released into cerebroventricular fluid in animal models of stroke (36), whereas drugs that block the “glycine site” of the NMDA receptors prevent stroke damage (37). Thus, it’s tempting to hypothesize that pharmacological inhibition of the enzymatic synthesis of D-serine would lead to reduced neurodegeneration. We describe herein the structural and functional characterization of recombinant serine racemase as well as its inhibition by chelators of divalent cations, thiol-reactive reagents, and serine analogues. Unexpectedly, isomerization of L-Ser into D-Ser was strictly dependent on the presence of divalent cations, since the addition of EDTA almost completely abrogated the enzyme activity (Fig. 2). Recombinant SR was active in the absence of any added divalent cations, most likely due to the saturation of...
the calcium binding site(s) of the enzyme during its folding inside *E. coli* or during the purification procedures. Significantly, the addition of both Ca$^{2+}$ and Mn$^{2+}$ to the reaction mixtures resulted in augmented synthesis of d-Ser in a clear reflection that this binding was physiologically relevant. Cystamine was found to be a potent inhibitor of SR, indicative that at least one sulfhydryl group is necessary for enzyme activity.

Several homogeneous PLP-containing alanine racemases of microbial origin are very effectively inhibited by alanine-phosphonic acid (25, 26); in fact, it is a time-dependent inactivator of all purified Gram-positive bacterial alanine racemases that have been tested. Additionally, the crystallographic complex of alanine phosphonic acid bound to the active site of *Bacillus stearothermophilus* alanine racemase was recently solved at 1.6-Å resolution (38). With that in mind and due to the potential therapeutic importance of developing novel SR inhibitors, we undertook the synthesis of the phosphonic acid analogue of serine (1-amino-2-hydroxyethane phosphonic acid). This effort was fruitless, since serine phosphonic acid failed to inhibit SR in the low millimolar range (Fig. 3). When working with a constant concentration of 10 mM substrate L-serine in the assay, only L-Ser-O-sulfate, L-cycloserine, and L-cysteine significantly inhibited the racemization reaction, whereas the other 13 serine structural analogues failed to diminish the synthesis of D-Ser when tested at 2 mM (Fig. 3). Consequently, successful therapeutic inhibition of SR will most likely befall by drugs structurally distinct from the substrate L-serine. In this regard, due to the direct binding and activation of SR by Ca$^{2+}$, compounds that lower intracellular calcium concentration might be promising agents against NMDA receptor overexcitation.

Significantly, L-cycloserine is widely used as a selective inhibitor of various PLP-containing enzymes, including serine palmitoyltransferase, the enzyme that catalyzes the first step of sphingolipid biosynthesis, using concentrations where serine racemase itself is also affected (39, 40). Inhibition by L-cysteine and by L-cycloserine was attained due to the direct reaction of the Ser analogues with the pyridoxal phosphate in the active site of the enzyme, as observed for the spectral changes observed in the visible region (Fig. 4). This spectral shift has been observed both in free (41, 42) and enzyme-bound PLP (27, 43) when reacting with L-cysteine and has been ascribed to the appearance of a stable complex containing a thiazolidine ring.

**FIG. 7.** Thermal denaturation profiles of recombinant SR in the presence of Ca$^{2+}$ or EDTA (A) and binding of $^{45}$Ca$^{2+}$ to recombinant SR (B). A, a 1.78 mM solution of SR in 20 mM Tris, 20 mM Mops, pH 7.8, 20 mM NaCl, 1 mM DTT was incubated with either 1 mM Ca$^{2+}$ or 1 mM EDTA, and the ellipticity was recorded at 220 nm in the 25–85 °C range. The samples were heated 1 °C/min. B, approximately 10 μg of recombinant SR were run in a SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane. A control gel stained with Coomassie Blue is also shown. The direct binding of $^{45}$Ca$^{2+}$ and the detection of the radioactivity signal was performed as described under “Materials and Methods.”

**FIG. 8.** Gel filtration chromatography of recombinant SR. A, elution profile of serine racemase in a Superdex HR200 column in the presence of 1 mM Ca$^{2+}$. The void volume is represented by a small bump appearing at approximately 7.8 ml after injection. The tetrameric and dimeric forms of SR elute at 11.4 and 12.7 ml, respectively. B, calibration of the Superdex HR200 column using a set of molecular mass standards as described under “Materials and Methods.” The elution position of the dimeric SR was interpolated in the calibration line (dashed lines). The proteins used to construct the calibration line include apoferritin (443 kDa), aldolase (158 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and myoglobin (17 kDa).
FIG. 9. Effect of glutamate, kainate, and the calcium ionophore A23187 in the synthesis of d-serine by serine racemase in astrocytes in culture (A) and in the protein levels of d-Ser (B). The glial cells were obtained from rat cortex, selected, and grown for 2 weeks as described under “Materials and Methods.” Incubation was performed in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium supplemented with 300 μM glutamate, 100 μM kainate, or 500 nM calcium ionophore A23187 for 24 h. The extracellular cell medium was then harvested, and the amount of d-Ser synthesized was determined by HPLC using Marfey reagent. A, the synthesis of d-Ser in the presence of glutamate, kainate, or A23187 was always compared with nonstimulated cells. In separate experiments, the same medium was incubated with increasing concentrations of d-Ser (up to 100 μM) in the absence of cells to obtain a calibration curve. B, in order to rule out the possibility that the increased d-Ser levels were due to changes in the expression levels of the enzyme, the amount of SR within astrocytes was also determined by immunodetection with an anti-serine racemase antibody.

The most significant finding of this work lies in the fact that SR activity is modulated by the calcium levels both in vitro (Fig. 2) and in vivo (Fig. 9). We have demonstrated that SR heterologously expressed in E. coli is active with levels of d-Ser synthesis that compare with those obtained for the protein obtained from mammalian cells (17) (Figs. 1 and 5). The recombinant SR was dimeric in equilibrium with the tetrameric form (Fig. 8); the efficient insertion of the PLP cofactor within the active site pocket and the proper folding of the enzyme were confirmed by the absorption spectrum in the visible region and fluorescence spectral changes (Fig. 6), and thermal stability in the presence of added calcium. Additional support for the direct binding of calcium to serine racemase was obtained using 45Ca2+ followed by autoradiography (Fig. 7).

Astrocytes enwrap synaptic terminals where they exert an active role as modulators of neurotransmission. Interestingly, whereas a role for astrocyte-derived d-Ser in neuronal glutamatergic transmission has been demonstrated (5–8, 30), the regulation of d-Ser synthesis by glial cells by glutamate or kainate is a less known process. Prior to the discovery of the existence of serine racemase, Snyder and co-workers (7) used exogenous d-[3H]serine to load astrocytes and evaluated the effect of added glutamate, kainate, and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate in the extracellular efflux of radioactive serine. Since the secretion of d-Ser in this case is unlikely to originate from SR-mediated isomerization of L-Ser into d-Ser, interpretation of this piece of data is somehow ambiguous. We show herein that not only the calcium ionophore A23187, but also glutamate and kainate, regulate the synthesis of d-Ser by glial cells unambiguously (Fig. 9). According to the in vitro calcium activation observed for recombinant SR, synthesis and secretion of d-Ser by the astrocytes in the presence of 500 nM A23187 very likely reflect the intracellular activation of the enzyme upon calcium entry. Under these circumstances, we were able to observe the synthesis of 21 μM d-Ser. Considering the results obtained with the recombinant enzyme, this increase in the extracellular d-Ser detected in astrocyte cultures in the presence of A23187 might be due to the direct binding of Ca2+ to the enzyme, rather than an indirect mechanism of activation of d-Ser release in the presence of the divalent cation. However, although our in vivo results show the same trend as the data obtained with the recombinant enzyme, we cannot rule out the modulation of SR activity by certain kinases or phosphatases that might be regulated by calcium within astrocytes. Finally, the levels of d-Ser that could be observed in the extracellular medium after glutamatergic activation of intracellular SR lay in the 15–25 μM range, a concentration high enough to activate the NMDA receptor (13), reinforcing the biological significance of our findings. Our observation that serine racemase activity in primary astrocytes appears to be regulated by glutamate could be considered a new example of bidirectional communication among neuron and glial cells (44). Thus, neuron-released glutamate would modulate SR activity that, in turn, affects NMDA signaling.

Acknowledgments—We thank Dr. Paloma Martinez-Ruiz for help during the synthesis of the phosphonic acid analogue of l-serine, Dr. Sonsoles Hortelano for performing the detection of nitrosylated serine racemase, and Teresa Gómez del Pulgar for assistance in culturing primary astrocytes. We are also indebted to Dr. Solomon H. Snyder (Johns Hopkins University) for providing the serine racemase cDNA.

REFERENCES
1. Hashimoto, A., Nishikawa, T., Hayashi, T., Fujii, N., Harada, K., Oka, T., and Takahashi, K. (1995) FEBS Lett. 389, 33–36.
2. Hashimoto, A., Oka, T., and Nishikawa, T. (1995) Neuroscience 66, 635–643.
3. Hashimoto, A., Kumashiro, S., Nishikawa, T., Oka, T., Takahashi, K., Mito, T., Takashima, S., Doi, N., Mizutani, Y., Yamazaki T., Kaneko, T., and Ootomo, E. (1993) J. Neurochem. 61, 348–351.
4. Dunlop, D. S., Neidle, A., McHale, D., Dunlop, D. M., Lajtha A. (1986) Biochem. Biophys. Res. Commun. 141, 21–32.
5. Wolosker, H., Sheth, K. N., Takahashi, M., Mokhet, J. P., Brady, R. O., Jr., Ferris, C. D., and Snyder, S. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 721–725.
6. Wolosker, H., Blackshaw, S., and Snyder, S. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 13409–13414.
7. Schell, M. J., Milliver, M. E., and Snyder S. H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 3948–3952.
8. Schell, M. J., Brady, R. O., Milliver, M. E., and Snyder S. H. (1997) J. Neurosci. 17, 1604–1615.
9. Yasuda, E., Ma, N., and Semba, R. (2001) Neurosci. Lett. 299, 162–164.
10. Kleckner, N. W., and Dingledine R. (1988) Science 241, 835–837.
11. Wibulski, M. T., Fadda, M., Mazzetta, J., Lazarewicz, J. W., and Costa, E. (1989) Neurupharmacology 28, 447–452.
12. Paudice, P., Gemignani, A., and Raiteri, M. (1998) Eur. J. Neurosci. 10, 2934–2944.
13. Matsui, T., Sekiguchi, M., Hashimoto, A., Tomita, U., Nishikawa, T., and Wada, K. (1995) J. Neurochem. 63, 454–458.
14. Galli, A., Franchi-Micheli, S., Mori, F., Luzzi, S., and Zilleti, L. (1990) Neuropharmacology 29, 145–150.
15. Mokhet, J. P., Parent, A. T., Wolosker, H., Brady, R. O., Jr., Linden, D. J., Ferris, C. D., Rogawski, M. A., and Snyder, S. H. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 4926–4931.
16. De Miranda, J., Santos, A., Engeldinger, S., Wolosker, H. (2000) Gene (Amst.) 256, 183–188.
17. Panizzi, R., De Miranda, J., Ribeiro, C. S., Engeldinger, S., Wolosker, H. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 5294–5299.
18. Rodriguez-Crespo, I., Gerber, N. C., Ortiz de Montelano, P. R. (1996) J. Biol. Chem. 271, 11462–11467.
19. Perera, A., Hollo, M., Tusnady, G., and Fasman, G. D. (1991) Protein Eng. 4, 669–679.
20. Martinez del Puoz, A., Merola, M., Ueno, H., Manning, J. M., Tanizawa, K., Nishimura, K., Soda, K., and Ringe, D. (1989) J. Biol. Chem. 264, 17784–17789.
21. Szokan, G., Mezo, G., and Hudecz F. (1988) J. Chromatogr. 444, 115–122
22. Lejczak, B., Kafarski, P., Soroka, M., and Mastalerz, P. (1984) Synthesis 577–580
23. Blázquez, C., Galve-Roperh, I., and Guzmán, M. (2000) FEBS J. 14, 2315–2322
24. Jaffrey, S. R., Erdjument-Bromage, H., Ferris, C. D., Tempst, P., Snyder, S. H. (2001) Nat. Cell Biol. 3, 193–197
25. Badet, B., and Walsh, C. (1985) Biochemistry 24, 1333–1341
26. Badet, B., Inagaki, K., Soda, K., and Walsh, C. T. (1986) Biochemistry 25, 3275–3282
27. Liu, J.-Q., Dairi, T., Itoh, N., Kataoka, M., Shimizu, S., and Yamada, H. (1998) J. Biol. Chem. 273, 16678–16685
28. Gallagher, D. T., Gilliland, G. L., Xiao, G., Zondlo, J., Fisher, K. E., Chinchilla, D., and Eisenstein, E. (1998) Structure 6, 465–475
29. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, Kluwer Academic/Plenum Publishers, New York
30. Baranano D. E., Ferris, C. D., and Snyder, S. H. (2001) Trends Neurosci. 24, 99–106
31. Lee, J.-M., Grabb, M. C., Zipfel, G. J., and Choi, D. W. (2000) J. Clin. Invest. 106, 723–731
32. Choi, D. W. (1992) J. Neurobiol. 23, 1261–1276
33. Atlante, A., Calissano, P., Bobba, A., Giannattasio, S., Marra, E., Passarella, S. (2001) FEBS Lett. 497, 1–5
34. Plaitakis, A., and Shashidharan, P. (2000) J. Biol. Chem. 275, 25–35
35. Obrenovitch, T. P. (1999) Ann. N. Y. Acad. Sci. 896, 273–286
36. Lo, E. H., Pierce, A. R., Matsumoto, K., Kato, T., Evans, C. J., and Newcomb, R. (1998) Neuroscience 83, 449–455
37. Wojciech, D., and Parsons, C. G. (1998) Pharmacol. Rev. 50, 597–664
38. Stamper, G. F., Morillo, A. A., and Ringe, D. (1998) Biochemistry 37, 10438–10445
39. Shimabukuro, M., Higa, M., Zhou, Y. T., Wang, M. Y., Newgard, C. B., and Unger, R. H. (1998) J. Biol. Chem. 273, 32487–32490
40. Sundaram, K. S., and Lev, M. (1984) J. Neurochem. 42, 577–581
41. Matsuo, Y. (1957) J. Biol. Chem. 232, 6042–6049
42. Schirch, L. V., and Mason, M. (1962) J. Biol. Chem. 237, 2578–2581
43. Gallo, V., and Ghiani, C. A. (2000) Trends Pharmacol. Sci. 21, 252–258