



Gabapentin and (S)-pregabalin decrease intracellular D-serine concentrations in PC-12 cells

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HIGHLIGHTS

- ▶ Gabapentin decreases intracellular D-serine, $IC_{50} = 3.40 \pm 0.29 \mu\text{M}$.
- ▶ (S)-pregabalin decreases intracellular D-serine, $IC_{50} = 3.38 \pm 0.21 \mu\text{M}$.
- ▶ Gabapentin and (S)-pregabalin do not affect serine racemase expression.

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ABSTRACT

The effects of gabapentin (GBP) and (S)-pregabalin (PGB) on the intracellular concentrations of D-serine and the expression of serine racemase (SR) in PC-12 cells were determined. Intracellular D-serine concentrations were determined using an enantioselective capillary electrophoresis assay with laser-induced fluorescence detection. Increasing concentrations of GBP, 0.1–20 μM , produced a significant decrease in D-serine concentration relative to control, $22.9 \pm 6.7\%$ at 20 μM ($*p < 0.05$), with an IC_{50} value of $3.40 \pm 0.29 \mu\text{M}$. Increasing concentrations of PGB, 0.1–10 μM , produced a significant decrease in D-serine concentration relative to control, $25.3 \pm 7.6\%$ at 10 μM ($*p < 0.05$), with an IC_{50} value of $3.38 \pm 0.21 \mu\text{M}$. The compounds had no effect on the expression of monomeric-SR or dimeric-SR as determined by Western blotting. The results suggest that incubation of PC-12 cells with GBP and PGB reduced the basal activity of SR, which is most likely a result of the decreased Ca^{2+} flux produced via interaction of the drugs with the $\alpha_2\text{-}\delta$ subunit of voltage-gated calcium channels. D-Serine is a co-agonist of the N-methyl D-aspartate receptor (NMDAR) and reduced D-serine concentrations have been associated with reduced NMDAR activity. Thus, GBP and PGB may act as indirect antagonists of NMDAR, a mechanism that may contribute to the clinical effects of the drugs in neuropathic pain.

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1. Introduction

Gabapentin, 1-(aminomethyl) cyclohexane acetic acid (GBP) and the structurally related (S)-pregabalin, (S)-3-(aminomethyl)-5-methylhexanoic acid (PGB), are used in the treatment of a wide-range of neuropathic pain conditions including diabetic neuropathy [1], postherpetic neuralgia [16], migraine and pain associated with cancer and multiple sclerosis [4]. Both compounds have similar pharmacological activity and are assumed to produce these effects via the same mechanism of action [19].

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GBP, the initial and more studied of the two compounds, was developed as GABA mimetic and the compound is active at GABA_B receptors, but not GABA_A receptors [11,23]. While GABA_B receptor activation has been associated with some of the therapeutic actions of GBP, it does not appear to be responsible for the drug's analgesic effects [11,15,23]. These effects have been associated with interaction with $\alpha_2\text{-}\delta$ subunit of voltage-gated calcium channels ($\text{Ca}_v\alpha_2\text{-}\delta$) and the resulting reduction in calcium (Ca^{2+}) influx [24]. It has also been reported that GBP reduces neuropathic pain responses by reducing hyperalgesia and allodynia via antagonistic activity at the N-methyl D-aspartate receptor (NMDAR) and Ca^{2+} channels in CNS and that D-serine (D-Ser), a NMDAR co-agonist, reverses the antihyperalgesic effect of GBP [15,19].

We now report the initial study of the effect of GBP and PGB on intracellular D-serine concentrations in the PC-12 cell line. The study is based upon the assumption that GBP and PGB associated decreases in the intracellular concentration of Ca^{2+} will decrease the activity of serine racemase (SR) the primary source of

endogenous D-serine. SR is a Ca^{2+} -dependent enzyme and previous studies have demonstrated that increased intracellular Ca^{2+} results in increased D-Ser production [6,8] while decreased Ca^{2+} results in decreased D-Ser production [2,9]. The current study was conducted using the PC-12 cell line which has been previously shown to express $\text{Ca}_v\alpha_2\text{-}\delta$ calcium channels [7,27] and monomeric and dimeric forms of SR, m-SR and D-SR [20]. The effects of GBP and PGB were assessed through the determination of relative changes in intracellular D-Ser concentrations using a previously validated enantioselective capillary electrophoresis – laser-induced fluorescence assay and SR expression using Western blotting technique [20]. The data demonstrate for the first time that in PC-12 cells, incubation with GBP and PGB decreased intracellular D-Ser concentrations in a concentration-dependent manner without affecting SR expression. The results suggest that the drugs attenuate SR activity and that this effect may represent a potential therapeutic mechanism of action of these drugs in the treatment of neuropathic pain.

2. Materials and methods

2.1. Materials

D-Serine (D-Ser), D-arginine (D-Arg), GBP, PGB, 2-hydroxypropyl- β -cyclodextrin (HP- β -CD), acetonitrile (ACN) and fluorescein isothiocyanate (FITC) were obtained from Sigma–Aldrich (St. Louis, MO). De-ionized water was obtained from a Milli-Q system (Millipore, Billerica, MA). All other chemicals used were of analytical grade.

2.2. Maintenance and treatment of cell lines

The PC-12 pheochromocytoma cell line was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI-1640 supplemented with 1 mM HEPES buffer, 10% horse serum, 5% FBS, 1% sodium pyruvate, 5% L-glutamine and 1% penicillin/streptomycin. RPMI-1640, fetal bovine serum (FBS), sodium pyruvate (0.1 M), L-glutamine (0.2 M) and penicillin/streptomycin solution (containing 10,000 units/ml penicillin and 10,000 $\mu\text{g}/\text{ml}$ streptomycin) were obtained from Quality Biological (Gaithersburg, MD), and HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer [1 M, pH 7.4] was obtained from Mediatech Inc. (Manassas, VA).

2.3. Incubation of PC-12 cells with GBP and PGB

The effect of GBP and PGB on intracellular D-Ser concentration was determined using a previously described protocol [20]. In brief, PC-12 cells were seeded on 100 mm \times 20 mm tissue culture plates and maintained at 37 °C under humidified 5% CO_2 in air until they reached >70% confluence. The original media was replaced with media containing the test compounds; the plates were incubated for an additional 36 h, the medium removed, and the cells collected for analysis. All of the studies were done in triplicate on two separate days. The GBP and PGB concentrations used in this study were chosen based on the previously reported concentration ranges that inhibited voltage-activated Ca^{2+} current in neurons; for the GBP studies the concentrations were: 0.1, 0.5, 1.0, 10.0, 20.0 μM [21] and for PGB studies the concentrations were 0.1, 0.50, 1.0, 2.0, 5.0, 10.0 μM [12].

2.4. Determination of intracellular D-Ser concentrations

Intracellular D-Ser concentrations were measured using a previously described and validated capillary electrophoresis-laser

induced fluorescence (CE-LIF) analysis performed using a P/ACE MDQ system equipped with a laser-induced fluorescence detector (Beckman Instruments, Fullerton, CA) [20]. In brief, at the completion of the incubations, the cells were collected, centrifuged, and the supernatant discarded. The cell pellet was resuspended in 1.00 ml of water, and 0.05 ml of D-Arg [100 μM in water] was added as internal standard, followed by 4.0 ml of acetonitrile. The resulting suspension was sonicated for 20 min, centrifuged for 15 min at 2500 $\times g$ at 4 °C and the supernatant collected and stream dried under nitrogen. The residue was dissolved in 0.9 ml of borate buffer [80 mM, pH 9.3] followed by 0.1 ml of FITC solution (3 mg/ml in acetone) and the resulting solution was placed in darkness for 12 h at room temperature. The samples were analyzed using an uncoated fused-silica capillary (50 μm I.D., effective length 50 cm), a running buffer composed of 500 μM HP- β -CD solution prepared in borate buffer [80 mM, pH 9.3] and detection at $\lambda = 488$ nm (excitation) and $\lambda = 520$ nm (emission). Quantification was accomplished using area ratios calculated for FITC-D-Ser with FITC-D-Arg as the internal standard. Calibration standards were assayed before the analyses performed in this study to ensure that the analytical method was performing as previously validated [20]. In this assay, the limit of detection (LOD) and limit of quantitation (LOQ) for D-Ser were 0.1 and 0.25 μM , respectively, the linearity was $r^2 = 0.998$ established between 0.25 and 100 μM and the method was reproducible with %CV values ranging between 0.7% and 2.7% (interday, $n = 3$). Relative migration factors of D-Ser and L-ser were calculated relative to the migration time of D-Arg (internal standard), calculated using 10 experiments per day over 3 days ($n = 30$). The average relative migration factor of D-Ser was 1.02 ± 0.02 , %CV = 2.36, and for L-ser the average was 1.05 ± 0.03 , %CV = 2.46.

2.5. Measurement of monmeric-SR (m-SR) and dimeric-SR (d-SR) expression by Western blotting

The expression of m-SR and d-SR in PC-12 cells was determined using a previously described procedure [20]. The primary antibody for D-SR was obtained from Santa Cruz Biotechnology, and the antibody that recognizes both m-SR and d-SR was purchased from Abcam, Inc. (Cambridge, MA). The primary antibody for β -actin was from Abcam. The antibodies were used at a dilution recommended by the manufacturer. Immunoreactive bands were detected using the ECL Plus Western Blotting Detection System (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) and quantification was accomplished by volume densitometry using ImageJ software (National Institutes of Health, Bethesda, MD) and normalization to β -actin.

2.6. Statistical analysis

The effect of the test compounds on intracellular D-Ser concentration (“response”) is reported as ‘average percent change \pm standard deviation’. The “response” versus drug concentration sigmoidal dose-response curves (IC_{50} curves) were determined for each of the 6 repeated sets using the ‘non-linear regression (curve fit)’ model contained within the Prism 4 software package (GraphPad Software, Inc. La Jolla, CA) running on a personal computer. The statistical significance of the concentration dependent effects on response for each of the drugs was determined using ANOVA for repeated measures with a 2 \times 6 model. A $p < 0.05$ was set for statistical significance and the analyses were performed using Systat version 10.2 software (SYSTAT Software, Inc. www.systat.com).

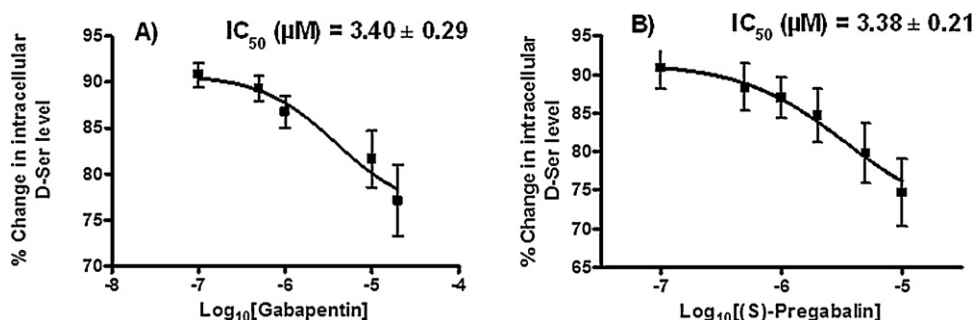


Fig. 1. The effect of increasing concentration of gabapentin (GBP), 0.1–20 μ M, and (S)-pregabalin (PGB), 0.1–10 μ M, on the intracellular concentration of D-Ser in PC-12 cells and PGB on the intracellular D-Ser concentration in PC-12 cells expressed as percent change relative to control; where: (A) effect observed with GBP; (B) effect observed with PGB.

3. Results

3.1. Effect of GBP on intracellular D-Ser concentrations and SR expression in PC-12 cells

The 36 h incubation of PC-12 cells with GBP (0.1–20 μ M) produced a statistically significant concentration-dependent reduction in intracellular D-Ser concentrations with a maximum decrease of $22.9 \pm 6.7\%$ ($*p < 0.05$) observed at a GBP concentration of 20 μ M (Fig. 1A). The plot of the average percentage decrease in D-Ser concentration versus GBP concentration produced a sigmoidal curve (Fig. 1A) with a calculated IC_{50} value of 3.40 ± 0.29 μ M. No change in SR expression was observed after treatment with GBP (Fig. 2A).

3.2. Effect of PGB on intracellular D-Ser concentrations and SR expression in PC-12 cells

The 36 h incubation of PC-12 cells with PGB (0.1–10 μ M) produced a statistically significant concentration-dependent reduction in intracellular D-Ser concentrations with a maximum decrease of

$25.3 \pm 7.6\%$ ($*p < 0.05$) observed at a PGB concentration of 10 μ M (Fig. 1B). The plot of the average percentage decrease in D-Ser concentration versus PGB concentration produced a sigmoidal curve (Fig. 1B) with a calculated IC_{50} value 3.38 ± 0.21 μ M. No change in SR expression was observed after treatment with PGB (Fig. 2B).

4. Discussion

The etiology of neuropathic pain has been associated with stimulation of the NMDAR in the dorsal horn, which produces a cumulative depolarization and a release of the magnesium block of the receptor [3,14]. Thus, it follows that one approach to the treatment of neuropathic pain is the reduction of NMDAR activity through the direct inhibition of NMDAR activity using NMDAR antagonists [5]. Indeed, NMDAR antagonists such as ketamine have been successfully used in the treatment of neuropathic pain and complex regional pain syndrome [18]. However, a recent meta-analysis of the clinical use of NMDAR antagonists in the treatment of neuropathic pain indicated that no significant conclusions can be made about the efficacy of the use of NMDAR antagonists [5].

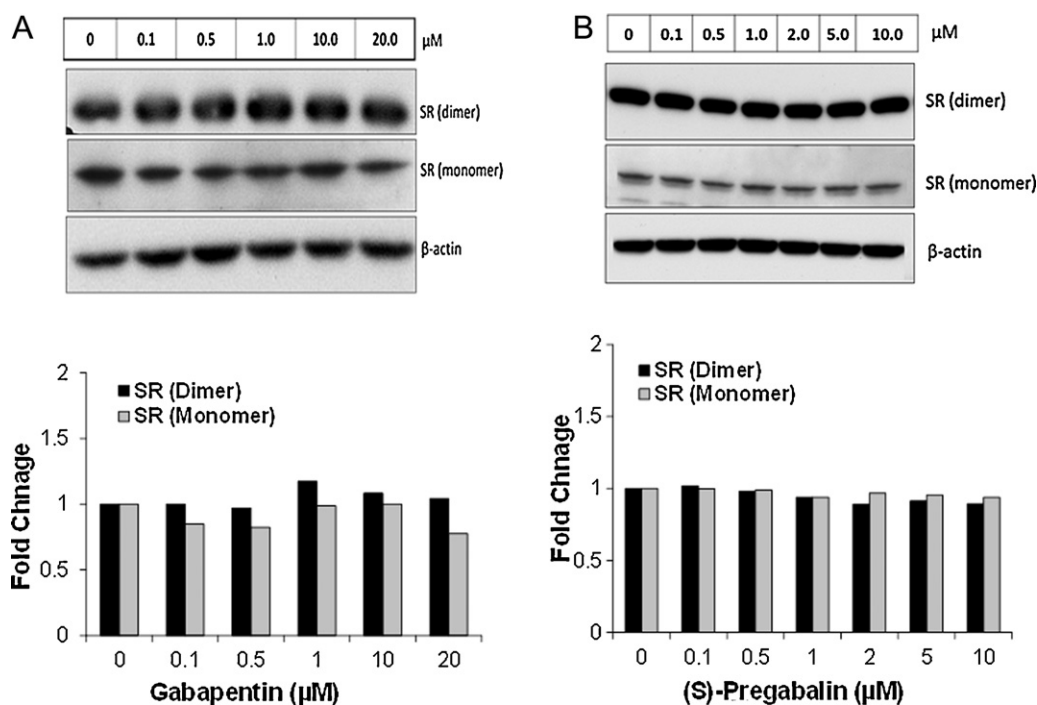


Fig. 2. Expression of SR protein after treatment with different concentrations of gabapentin and pregabalin for 36 h. (A) Western blot analysis in PC-12 cells with SR antibodies shows the dimeric and monomeric forms (top) after treatment with gabapentin. (B) Western blot analysis in PC-12 cells with SR antibodies shows the dimeric and monomeric forms (top) after treatment with (S)-pregabalin (top). (A and B) Relative SR expression after quantification and normalization with β -actin is shown (bottom).

A second approach to the treatment of neuropathic pain is the indirect inhibition of NMDAR activity through the reduction in endogenous D-Ser concentrations. This approach was suggested by the observation that endogenous D-Ser in the rostral anterior cortex of the rat is related to specific pain-related negative emotion [13]. Based upon this observation, the authors suggested that reducing D-Ser concentrations and, thereby, NMDAR activity, may be a new strategy for reducing chronic pain-induced emotional disturbance [13]. The reduction of D-Ser concentrations can be achieved through the direct inhibition of SR activity and current drug discovery programs are developing competitive and allosteric inhibitors of SR for the treatment of amyotrophic lateral sclerosis (ALS) and Alzheimer's disease [8].

Another approach to the inhibition of SR activity is suggested by the fact that SR is a pyridoxal-5'-phosphate-dependent enzyme that requires the binding of Ca^{2+} for activation [8,9]. Modulation of SR activity, determined by the efflux of D-Ser, has been achieved through the reduction of intracellular Ca^{2+} through the addition of chelating agents to the incubation media [26] or increased using the calcium ionophore, A23187 [6,9]. We have also recently shown that the treatment of PC-12 cells with *Escherichia coli* lipopolysaccharide (LPS) increased intracellular D-Ser [20], which is consistent with LPS induced Ca^{2+} influx, see for example [10,26]. Thus, this approach would involve the reduction of NMDAR activity via alterations in intracellular Ca^{2+} produced by effects at receptors and transporters involved in Ca^{2+} flux. This is the mechanism explored in this study.

In the current study, incubation of PC-12 cells with 25 μM GBP and 10 μM PGB produced a 25% reduction in intracellular D-Ser and no statistically significant difference was found between the effect of GBP and PGB ($p=0.88$). The magnitude of the reduction and the relative potency of the two compounds are consistent with the 25–30% reduction Ca^{2+} influx produced by GBP (25 μM) and PGB (2.5 μM) [12,21]. In this study, the IC_{50} values observed for the decrease in intracellular D-Ser concentration were the same for both GBP and PGB, 3.4 μM . These values differ from the IC_{50} values determined for the reduction of Ca^{2+} influx produced by GBP and PGB, 0.167 μM and 0.073 μM , respectively [17,21]. However, one might expect these values to differ as the latter experiments address a direct effect on Ca^{2+} influx, while the former an indirect consequence of the decrease in intracellular Ca^{2+} .

In our previous study of the effect of competitive inhibitors, substrate concentration and LPS on the expression and function of m-SR and D-SR in PC-12 cells we observed that in addition to an increase in intracellular D-Ser, incubation with LPS increased the expression of D-SR relative to m-SR, as determined by Western blotting analysis [20]. It was unclear from the data in the previous study whether this effect was due to increased/decreased SR protein synthesis, which has been associated with LPS treatment [29] or an alteration in the m-SR: D-SR equilibrium. Alterations in SR expression have also been reported after acute and chronic administrations of morphine [30,31] and ketamine [22,25]. Based upon these observations we determined the effect of incubation with GBP and PGB on m-SR and D-SR in PC-12 cells. No changes in the relative expression of these proteins were observed indicating that the reduction of intracellular Ca^{2+} via interaction with $\alpha_2\text{-}\delta$ subunit of voltage-gated calcium channels ($\text{Ca}_v\alpha_2\text{-}\delta$) does not affect signaling pathways associated with the expression of SR.

5. Conclusions

The data from this study demonstrate that GBP and PGB reduce the intracellular concentration of D-Ser and suggest that this effect occurs via the attenuation of the basal activity of SR. D-Ser is a co-agonist of the NMDAR and the results suggest that GBP and PGB are indirect antagonists of this receptor. Since the inhibition of NMDAR

has been associated with the treatment of neuropathic pain, the proposed mechanism may explain some of the clinical effects of GBP and PGB. An additional potential interconnection between GBP and the endogenous production of D-Ser by SR is suggested by the observations that GBP treatment prevents motoneuron degeneration in an in vitro model of amyotrophic lateral sclerosis (ALS) [23] and that endogenous D-Ser plasma concentrations are increased in ALS patients [28].

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