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

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HIGHLIGHTS

- Gabapentin decreases intracellular D-serine, $IC_{50} = 3.40 \pm 0.29\ \mu M$.
- (S)-pregabalin decreases intracellular D-serine, $IC_{50} = 3.38 \pm 0.21\ \mu M$.
- 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 $3.40 \pm 0.29\ \mu M$. 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 $3.38 \pm 0.21\ \mu M$. 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 α_2-δ 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.

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].

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 α_2-δ subunit of voltage-gated calcium channels ($Ca_v\alpha_2$-δ) 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

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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 $Ca_v\alpha_2$-δ 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 enantioslective 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 μg/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 × 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 × *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 λ = 488 nm (excitation) and λ = 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 ± 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 'nonlinear 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 2×6 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).