Effects of pregabalin on the activity of glutamate transporter type 3

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Editor’s key points

- The effect of pregabalin on neuronal glutamate transporter 3 (EAAT3) was measured.
- Voltage patch clamping of oocytes was used.
- Various inhibitors were applied along with pregabalin.
- Pregabalin increased EAAT3 activation.
- Protein kinase and phosphatidylinositol-3-kinase were implicated in this activation.

Background. Pregabalin, (S)-3-aminomethyl-5-methyl hexanoic acid, is a ligand for the α2δ subunit (a component of voltage-gated calcium channels) and has analgesic and anticonvulsant properties. Glutamate uptake by glutamate transporters may be a mechanism for these properties. We investigated the effects of pregabalin on the activity of the neuronal glutamate transporter type 3 (EAAT3).

Methods. EAAT3 was expressed in Xenopus laevis oocytes. Two-electrode voltage clamping was used to record membrane currents before, during, and after applying L-glutamate (30 μM) in the presence or absence of pregabalin. Currents were also measured in oocytes pretreated with a protein kinase C (PKC) activator (phorbol-12-myristate-13-acetate, PMA), PKC inhibitors (chelerythrine or staurosporine), or a phosphatidylinositol-3-kinase (PI3K) inhibitor wortmannin.

Results. The exposure of the oocytes injected with EAAT3 mRNA to serial concentrations of pregabalin (0.06–60 μM) significantly increased their responses to 30 μM L-glutamate. A kinetic study showed that pregabalin significantly increased Vmax without changing Km. Treatment of oocytes with PMA, pregabalin, or pregabalin plus PMA significantly increased transporter currents vs controls, but treatment with PMA plus pregabalin did not increase the responses further vs PMA or pregabalin alone. In addition, pretreatment of oocytes with two PKC inhibitors (chelerythrine or staurosporine), or inhibitor wortmannin, significantly reduced basal and pregabalin-enhanced EAAT3 activity.

Conclusions. Pregabalin increased EAAT3 activity and PKC and PI3K were involved. This may explain the analgesic effect of pregabalin in neuropathic pain.

Keywords: analgesics non-opioid; pain, neuropathic

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Pregabalin, (S)-3-aminomethyl-5-methyl hexanoic acid, is a recently developed analgesic, anxiolytic, and anticonvulsant agent.1 In clinical settings, pregabalin is used to control neuropathic pain in patients with diabetic neuropathy, neur- algia, or complex regional pain syndrome.1 2 Pregabalin has also been investigated as a neuroprotector after spinal cord injury in rats, where neuroprotection may be related to its anti-apoptotic and anti-inflammatory effects.3

Pregabalin is a lipophilic analogue of γ-aminobutyric acid (GABA), but it neither acts like GABA nor binds to GABA receptors. The α2δ subunit of the plasma membrane voltage-gated calcium channel is known to be a major site of pregabalin action.4 Its potent binding at this site reduces calcium influx to presynaptic nerve endings5 and also the release of several neurotransmitters, such as glutamate6 7 and norepinephrine,8 thereby attenuating postsynaptic excitability.

Although the α2δ subunit of the calcium channel blocker may be the primary target for pregabalin, it may possess additional mechanisms of action. It has been proposed that pregabalin has indirect effect on the GABA transporter 1 (GAT-1), although it does not bind to GABA receptors.9 GAT-1 protein is redistributed from intracellular locations to the cell membrane by prolonged exposure to pregabalin and GABA uptake in primary hippocampal neuronal cultures was increased.10 A recent study also suggested that prolonged exposure to pregabalin produces delayed allosteric enhancement of an unspecified voltage-activated potassium current in rat dorsal root ganglion neurons, possibly via the activation of protein kinase A.11 In the glutamate neurotransmitter system, pregabalin, as an anticonvulsant, decreased K+-evoked glutamate release from neocortical and hippocampal slices.5

However, the role of pregabalin effects on the glutamate transporter in terms of analgesic, anticonvulsant, and neuroprotective effects has not yet been investigated. Given this theoretical background, we evaluated the effect of pregabalin
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on EAAT3, a major neuronal glutamate transporter, expressed in Xenopus oocytes by using two-electrode voltage clamping, and investigated the involvements of protein kinase C (PKC) and phosphatidylinositol-3-kinase (PI3K), two intracellular signalling molecules.

Methods

Isolation and microinjection of Xenopus oocytes were done as described by Do and colleagues. Mature female Xenopus laevis frogs were purchased from Xenopus I (Ann Arbor, MI, USA), and fed with regular frog brittle twice weekly. Frogs were anaesthetized with 500 ml of 0.2% 3-amino benzoic acid ethyl ester (Sigma, St Louis, MO, USA) in water until unresponsive to painful stimuli (toe pinching). Operations for removing oocytes were performed on ice. A 5 mm long incision was made in the lower lateral abdominal quadrant, and then a lobule of ovarian tissue (containing ~150 oocytes) was removed and isolated manually from their surrounding follicles. Then, those follicles were immersed in calcium-free OR-2 solution (containing in mM: NaCl 82.5, KCl 2, MgCl2 1, HEPES 5, collagenase type Ia 0.1%, pH 7.5) to remove the vitelline membrane surrounding oocytes. Defolliculation was done by gentle shaking for ~2 h at 18°C in modified Barth's solution [containing in mM: NaCl 88, KCl 1, NaHCO3 2.4, CaCl2 0.41, MgSO4 0.82, Ca(NO3)2 0.3, gentamicin 0.1, HEPES 15, pH adjusted to 7.6].

The rat EAAT3 complementary DNA (cDNA) construct was provided by Dr M.A. Hediger (Brigham and Women’s Hospital, Harvard Institute of Medicine, Boston, MA, USA), and contained cDNA subcloned in a commercial vector (Blue-scriptSKm). Plasmids containing the glutamate transporter cDNA were linearized with the restriction enzyme NotI and messenger RNA (mRNA) was synthesized using an in vitro transcription kit (Ambion, Austin, TX, USA). The yield of the in vitro transcription product was quantified spectrophotometrically at 260 nm and diluted in sterile water. Oocytes were injected with 30 ng/30 nl mRNA using a nanoject injector (Drummond Scientific, Broomall, PA, USA) with a glass micropipette of tip diameter 17–20 μm. Before current recording was performed, individually injected oocytes were maintained for 3 days at 18°C in microtitre plates containing modified Barth’s solution. The incubation medium was changed daily and degenerating oocytes were discarded.

Electrophysiological recordings were performed at room temperature (~21–23°C). Microelectrodes were pulled in one stage from 10 μl capillary glass (Drummond Scientific Co.) on a micropipette puller. Tips were broken at a diameter of ~10 μm and filled with 3 M KCl. A single defolliculated oocyte was placed in a recording chamber (0.5 ml volume) and perfused with Tyrode’s solution [containing in mM: NaCl 150, KCl 5, CaCl2 2, MgSO4 1, dextrose 10, and HEPES 10 at pH 7.5] at a flow rate of 3 ml min⁻¹ for 4 min before currents were measured. Two recording electrodes (1–5 MΩ) were inserted into individual oocytes. A Warner Oocyte-clamp OC 725-C (Warner, Hamden, CT, USA) was used to voltage clamp each oocyte at −70 mV. Data acquisition and analysis were performed using a personal computer running OoClamp software. Oocytes that did not show a stable holding current of ≤1 μA were excluded from the analysis.

L-glutamate was diluted in Tyrode’s solution and superfused over clamped oocytes for 20 s at 3 ml min⁻¹. L-glutamate-induced inward currents were sampled at 125 Hz for 1 min, that is, 5 s at baseline, 20 s of L-glutamate application, and 35 s of washing period (conducted with Tyrode’s solution). Responses were quantified by integrating current traces and are reported as microCoulombs (μC), which reflect the total amount of glutamate transported. Each experiment was performed using oocytes from at least four different frogs.

Pregabalin (Lyrica®) was provided from Pfizer Inc. (Seoul, Republic of Korea). In the pregabalin-treated group, oocytes were incubated for 72 h with pregabalin (0.6, 1.8, 6, 18, 60, or 180 μM) before response to L-glutamate was measured. During this investigation, pregabalin was initially dissolved in dimethyl-sulphoxide (DMSO) and this concentrated stock solution was subsequently diluted with Tyrode’s solution to concentrations of 0.06, 0.18, 0.6, 1.8, 6, 18, or 60 μM. To study the concentration–response effect of pregabalin on EAAT3 activity, oocytes were exposed to 0 (control), 0.06, 0.18, 0.6, 1.8, 6, 18, or 60 μM pregabalin. To determine the effects of pregabalin 0.6 μM on the Km and Vmax, values of EAAT3 for glutamate, serial concentrations of L-glutamate (3, 10, 30, 100, or 300 μM) were used. In other experiments, 30 μM L-glutamate was used to induce glutamate transporter currents. To study the role of PKC on EAAT3 activity, oocytes were preincubated with 100 nM phorbol-12-myristate-13-acetate (PMA) for 10 min before recording. Some of the pregabalin-treated oocytes were exposed to PMA for 72 h in the manner as described above. To study the effects of PKC inhibitors on EAAT3 activity, oocytes and some of the pregabalin-pretreated oocytes were exposed to staurosporine (2 μM for 1 h) or to wortmannin (100 μM for 1 h). To investigate the role of PI3K on the regulation of EAAT3 activity by pregabalin, oocytes and pregabalin-treated oocytes were incubated with wortmannin (a PI3K inhibitor; 10 μM) for 1 h. Incubation with PMA, staurosporine, chelerythrine, or wortmannin was performed in a non-perfused dish containing these chemicals at the above-mentioned concentrations. Molecular biology reagents were obtained from Ambion, whereas other chemicals were from Sigma Aldrich Korea (Seoul, Republic of Korea), unless otherwise specified.

Analysis and tests of normality were performed using SPSS version 15.0 for Windows (SPSS, Chicago, IL, USA) and results are provided as means (±SD). Because batch-to-batch variability in oocyte response is common due to different EAAT3 expression levels, responses were normalized vs same-day controls for each oocyte batch. During data normalization procedure, all of the same-batch data are divided by the mean of the same-batch control. Then, the mean of the control subgroup becomes 1.00, and the data of the other subgroups can be expressed as the ratio vs control. Statistical analysis was performed using Student’s t-test or by analysis of variance followed by Bonferroni’s post hoc comparison as appropriate. P-values of <0.05 were considered significant.
Results

Oocytes microinjected with EAAT3 mRNA displayed inward currents after l-glutamate infusion, whereas those not injected with EAAT3 mRNA did not respond to l-glutamate administration (data not shown). Exposing oocytes injected with EAAT3 mRNA to serial concentration of pregabalin (0.06, 0.18, 0.6, 1.8, 6, 18, or 60 µM) increased their responses to 30 µM l-glutamate concentration dependently (Fig. 1). The EC_{50} of pregabalin for increasing EAAT3 response was 1.43 µM. Responses of oocytes to ≥0.06 µM pregabalin were significantly different from those of controls (conducted with Tyrode’s solution) and 0.6 µM pregabalin was chosen for further studies. In vehicle control experiments, 0.06% of DMSO (the highest final concentration used in dilution of pregabalin) had no effect upon current responses to glutamate [1.01 (0.19) µC for the control group vs 1.05 (0.34) µC for the DMSO-treated group, n=12; P>0.05].

The kinetic study showed that pregabalin significantly increased V_{max} from 1.83 (0.16) µC for controls to 2.46 (0.26) µC (n=25 in each group) without changing the K_{m} [3.86 (1.88) µM for controls to 8.75 (4.27) µM for the pregabalin group, P>0.05] (Fig. 2).

Treatment of oocytes with PMA, a PKC activator, or pregabalin 0.6 µM significantly increased the transporter currents [1.0 (0.13) for the control group (n=25) vs 1.32 (0.24) µC for the PMA group (n=17) and 1.41 (0.20) µC for the pregabalin group (n=21); P<0.05]. However, treatment with PMA plus pregabalin [1.51 (0.45) µC, n=17] did not increase responses more than PMA or pregabalin alone (Fig. 3).

Furthermore, pretreatment of oocytes with staurosporine or chelerythrine, two PKC inhibitors, significantly reduced pregabalin-enhanced EAAT3 activity [1.0 (0.16) µC for controls vs 0.78 (0.14) µC for staurosporine, n=12–19, P=0.00; 1.0 (0.05) µC for controls vs 0.85 (0.12) µC for chelerythrine, n=10–15, P=0.01] and abolished pregabalin-mediated increases in EAAT3 activity [1.41 (0.30) µC for pregabalin vs 1.13 (0.30) µC for pregabalin–staurosporine, n=12–19, P=0.02; 1.36 (0.32) µC for pregabalin vs 0.73 (0.13) µC for pregabalin–chelerythrine, n=10–15, P=0.00] (Fig. 4).

Oocytes pretreated with wortmannin showed significantly reduced basal EAAT3 activity [1.0 (0.18) µC for controls (n=17) vs 0.66 (0.14) µC for wortmannin (n=19), P=0.00] and pregabalin-enhanced EAAT3 activity [1.44 (0.16) µC for pregabalin (n=15) vs 0.80 (0.18) µC for pregabalin–wortmannin (n=19), P=0.00] (Fig. 5).
as a ‘high affinity’ system for glutamate transporters. Glutamate uptake mediated by glutamate transporter proteins located in the plasma membrane of glial cells and neurons is a high-affinity uptake system, which uses the 

$$\text{Na}^+$$-K$^+$ electrochemical gradient as a driving force. In the present study, EAAT3 exhibits specificity, in that it is present mainly in the cytosol with only about 20% at the plasma membrane and glutamate uptake is reportedly associated with rapid alterations in the trafficking of the glutamate transporter protein to the plasma membrane. The increased $V_{\text{max}}$ observed in this study suggests that exposure to pregabalin changes the location of EAAT3 from a cytoplasmic compartment to the plasma membrane.

In a previous study, EAAT3 activity and expression were demonstrated to be highly regulated by neuronal activity and by intracellular signalling pathways, including PKC and PI3K. Furthermore, PKC activation has been shown to increase EAAT3 activity and this effect was associated with the redistribution of EAAT3 from subcellular compartments to the plasma membrane. Another study also provided evidence that a particular PKC subtype, PKC$_{\alpha}$, specifically regulates the redistribution of EAAT3, in that pharmacological block of PKC$_{\alpha}$ prevented the PMA-induced increase in EAAT3 cell surface expression. The activation of PKC with PMA induced the formation of EAAT3–PKC$_{\alpha}$ complexes, which contributed to the stabilization of the transporter at the plasma membrane from intracellular stores and therefore increased the transport rate of glutamate. Our results show that PMA, a powerful PKC activator, and pregabalin do not act additively or synergistically to increase EAAT3 activity and this suggests that these two agents increase EAAT3 activity via the same pathway (redistribution).

Moreover, two PKC inhibitors, staurosporine (2 $\mu$M) and chelerythrine (100 $\mu$M), decreased basal EAAT3 activity and inhibited pregabalin-increased EAAT3 activity, respectively, suggesting that PKC may be associated with the mechanism whereby pregabalin enhances EAAT3 activity. These inhibitors act at different sites on PKC. More specifically, staurosporine occupies the ATP binding site, while chelerythrine acts on the negative catalytic domain of PKC.

PI3K is the primary enzyme involved in the synthesis of 3-phosphoinositides at the plasma membrane, which in turn recruit other effectors and regulate the trafficking of several membrane proteins. The implication of the PI3K signalling pathway in the activity of EAAT3 was recently confirmed by data showing that wortmannin, a PI3K inhibitor, significantly decreased EAAT3 membrane expression in biotinylation and immunoblotting experiments and induced clustering EAAT3 in the intracellular compartment in confocal microscopic analysis. In the present study, wortmannin decreased basal EAAT3 activity and abolished EAAT3 activity enhancement by pregabalin, suggesting that PI3K is also involved in the enhancement of EAAT3 activity by pregabalin.

Pregabalin does not bind to plasma proteins and is not hepatically metabolized in men; a previous study in healthy volunteers indicated its oral bioavailability to be $\sim$90%.

**Discussion**

Our findings indicate that pregabalin enhanced the activity of EAAT3, a major neuronal glutamate transporter, in a concentration-dependent (0.06–60 $\mu$M) manner by increasing $V_{\text{max}}$ and suggest that PKC and PI3K mediate this effect.

The reported affinities of glutamate uptake systems are preparation-dependent, and thus reported differences may be due to the different assays used to detect different glutamate transporter subtypes. In the present study, the $K_m$ value was around 4–9 $\mu$M, and this may be explained by the redistribution of EAAT3 from subcellular compartments to the plasma membrane.
Serum concentrations in patients receiving 600 mg of pregabalin were reported to range from 2.8 to 8.2 mg litre\(^{-1}\). In another study, the mean (range) plasma pregabalin concentrations associated with doses of 150 and 600 mg day\(^{-1}\) were 1.27 (0.29–2.84) and 4.88 (0.87–14.2) mg litre\(^{-1}\), respectively. Based on these previous investigations, oocytes in the current study were incubated for 72 h with pregabalin at a range of concentrations (0.01–10 mg litre\(^{-1}\) which correspond to 0.06–60 µM).

Pregabalin binds to the presynaptic Ca\(^{2+}\) channel α2δ subunit and selectively attenuates depolarization-induced Ca\(^{2+}\) influx. As a result, glutamate/aspartate release from excitatory amino acid (EAA) nerve terminals and the activation of AMPA heteroreceptors on noradrenergic nerve terminals are decreased. However, the interaction with Ca\(^{2+}\) channel α2δ subunit is not sufficient to account for the broad clinical spectrum of pregabalin effects. In the glutamate system, pregabalin significantly decreased cellular glutamate concentrations in the healthy rat forebrain. In addition, pregabalin decreased glutamate release at neocortical and hippocampal slices and at synapses in the entorhinal cortex, which are considered a critical site of seizures.

Glutamate, a major EAA neurotransmitter, causes excitotoxic neuronal injury when it accumulates extracellularly. Glutamate receptors have a rapid desensitization rate and this exposes the synapses to excessive glutamate concentrations. Furthermore, the uptake of glutamate through glutamate transporters contributes to the relocation of glutamate from extracellular to intracellular spaces and maintains extracellular glutamate concentrations within a physiological range. Of the five glutamate transporters (EAAT 1–5), EAAT3 is present at postsynaptic neuronal elements, whereas EAAT1 and EAAT2 are expressed in the astrocytes at their plasma membrane near the EAA synapses. EAAT1 and EAAT2 in astrocytes play key roles in the removal of glutamate from the synaptic space. Although EAAT3 makes a minor contribution to glutamate uptake from the synapse with a rather low expression level, it has also been shown to contribute efficiently to the clearance process.

EAAT3 may provide more than simple uptake and removal of glutamate from the extracellular space. In the hippocampus and cerebral cortex, neuronal uptake by EAAT3 plays a neuroprotective role, as these areas are not surrounded by astrocytic processes. In addition, EAAT3 contributes to neuroprotection by uptake of cysteine, which acts as a key substrate for the synthesis of glutathione, a major antioxidant. Thus, the contribution of the EAAT3 system could be emphasized in the investigation of dynamic glutamate uptake process.

Alterations in glutamate uptake have been implicated in the pathogenesis of neurological disorders, such as epilepsy, neuropathic pain, and neuroprotection. Changes in EAAT3 activity and expression have been shown to be correlated with epilepsy in experimental models. Furthermore, blocking EAAT3 expression increased seizure occurrence in rats, whereas increased glutamate uptake via enhanced EAAT3 activity was found to have an anticonvulsant effect. In addition, both expression and glutamate uptake activity of spinal neuronal and glial glutamate transporters are altered after peripheral nerve injury, thus contributing to neuropathic pain behaviours in rats with chronic constriction nerve injury. After chronic constriction nerve injury, spinal glutamate transporter showed a biphasic change, with an initial up-regulation followed by down-regulation.

Glutamate uptake through increased glutamate transporter activity in neurons and glia is also considered to account for brain protection. Since the affinity of the transporter for cysteine is equivalent to its affinity for glutamate, it has been proposed that EAAT3 acts via cysteine uptake to provide protective action against oxidative stress-related neurodegeneration. These mechanisms may explain the neuroprotective effect of pregabalin.

A few limitations of this study should be noted. First, further studies of EAAT 1 and 2 and in comparison with EAAT3 are needed to identify the selectivity of pregabalin before focusing exclusively on its effects on the EAAT3. Secondly, a study using surface labelling is needed to confirm that the observed increase in transporter currents is due to an increase in the expression levels of EAAT3 at the cell surface. In summary, pregabalin increased EAAT3 activity and PKC and PI3K were found to contribute to this effect. In terms of clinical implications, increased EAAT3 activity induced by pregabalin appears to be related to its analgesic effect against neuropathic pain.

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**Declaration of interest**

None declared.

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