Presynaptic inhibitory actions of pregabalin on excitatory transmission in superficial dorsal horn of mouse spinal cord: further characterization of presynaptic mechanisms

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Abstract

Pregabalin is widely used as an analgesic for the treatment of neuropathic pain. In the present experiments using mouse spinal slices, we recorded electrically-evoked glutamatergic excitatory postsynaptic currents (eEPSCs) from superficial dorsal horn neurons. Pregabalin reduced the amplitude of eEPSCs, and increased the paired pulse ratio. Pregabalin also inhibited the frequency of spontaneously occurring miniature EPSCs without affecting their amplitude. Partial ligation of the sciatic nerve increased the expression of the calcium channel α2δ-1 subunit, and increased the presynaptic inhibitory action of pregabalin. Intrathecal injection of antisense oligodeoxynucleotide against the α2δ-1 subunit, decreased the expression of α2δ-1 mRNA in the spinal dorsal horn, and decreased pregabalin's action. These results provide further evidence that pregabalin exerts its presynaptic inhibitory action via binding with the α2δ subunit in a state-dependent manner. Furthermore, presynaptic actions of pregabalin were attenuated in knockout mice lacking the protein syntaxin 1A, a component of the synaptic vesicle release machinery, indicating that syntaxin 1A is required for pregabalin to exert its full presynaptic inhibitory action. These observations might suggest that direct and/or indirect interactions with the presynaptic proteins composing the release machinery underlie at least some part of pregabalin’s presynaptic actions.

Keywords: Pregabalin; Release Machinery; Excitatory Synaptic Transmission; Tight-seal Whole-Cell Recording; Syntaxin 1A Knockout; Spinal Dorsal Horn

Highlights

1. Antisense ODN reduced Ca channel α2δ expression and weakened pregabalin's action.

2. Nerve injury increased Ca channel α2δ expression and augmented pregabalin's action.

3. Pregabalin's presynaptic actions were attenuated in syntaxin 1A knockout mice.

4. Syntaxin 1A is necessary for pregabalin to exert its full presynaptic inhibition.

1. Introduction

Pregabalin (PGB) is S-enantiomer of racemic 3-isobutyl GABA [21], having structure and actions similar to gabapentin. PGB has been used effectively for the treatment of neuropathic pain associated with diabetic peripheral neuropathy, postherpetic neuralgia and other conditions [13].

The analgesic effects of PGB are shown to be mediated by its binding with the $\alpha2\delta$ subunit of voltage-gated calcium channels [4, 9, 20]. This binding inhibits the influx of Ca^{2+} into the presynaptic terminals, which leads to a subsequent reduction in the release of neurotransmitters such as glutamate and substance P [3, 8].

Experiments on cultured hippocampal neurons loaded with fluorescent dye in synaptic vesicles, have shown that calcium-independently-occurring release of fluorescent dye is inhibited by PGB [10]. Additionally, the frequency of spontaneously occurring miniature excitatory postsynaptic currents, which is independent of presynaptic Ca^{2+} influx, is inhibited by gabapentin in the entorhinal cortex [2]. Thus, it has been proposed that PGB inhibits transmitter release by reducing presynaptic Ca^{2+} influx, and also by modulating transmitter release machinery downstream of Ca^{2+} influx.

Several electrophysiological studies with various results have been reported in regards to the effects of PGB and gabapentin on synaptic transmission in the spinal dorsal horn [11, 12, 15, 16]. It has been reported that gabapentin presynaptically inhibits glutamatergic synaptic transmission in normal naive rats [15]. Others have reported that the presynaptic inhibitory effects of gabapentin take place in hyperalgesic conditions but not in normal conditions [12, 16]. With regard to such state-dependent effects of gabapentin and PGB, several studies have suggested the expression level of the calcium channel $\alpha2\delta$ subunit in spinal dorsal root ganglion and in the spinal dorsal horn as one of the possible mechanisms [4, 9, 20].

In the present experiments using electrophysiological recordings, we investigated the effects of PGB on excitatory synaptic transmission in the superficial dorsal horn in an attempt to understand the molecular mechanisms underlying the state-dependent presynaptic inhibitory actions of PGB. Furthermore, using knockout mice lacking syntaxin 1A, which is a member of the SNARE proteins, and which forms presynaptic release machinery with SNAP-25, synaptobrevin and other presynaptic proteins, we tried to obtain clues to an understanding about whether and how PGB inhibits excitatory synaptic transmission by acting on the presynaptic release machinery.

2. Materials and Methods

2.1. Animals

Animal experimental procedures were reviewed and approved by the institutional animal care and use committees at Dokkyo Medical University. The care and use of the animals were in accordance with the guidelines of the International Association for the Study of Pain [22]. Syntaxin 1A knockout mice were generated as previously described [5], and backcrossed into the C57BL/6J genetic background. Experiments were performed on 68 male mice of age 6–8 weeks.

2.2. Partial ligation of the sciatic nerve and assessment of mechanical allodynia

Mice were anesthetized with halothane for the nerve-ligation surgery and for the sham surgery. The left sciatic nerve was partially ligated according to the protocol described by Seltzer et al [14]. The mechanical sensitivity of the hindpaw was assessed with a Von Frey filament of #3.61, which is equivalent to 0.41 g force. The number of foot withdrawals to 10 repetitive stimuli was recorded.

2.3. Preparation of spinal cord slices

Animals were anaesthetized with ketamine (100 mg / kg) and xylazine (10 mg / kg) intraperitoneally, and segments at the lumbosacral level of the spinal cord were removed. A microslicer (Dosaka EM) was used to cut transverse slices (350–450 μm) in Krebs' solution at 4 °C. The Krebs' s

solution contained the following ingredients (in mM): NaCl, 113; KCl, 3; $NaHCO_3$, 25; NaH_2PO_4, 1; $CaCl_2$, 2; $MgCl_2$, 1; D-glucose, 11.

2.4. Tight-seal whole-cell recordings

The spinal slices were mounted in a recording chamber on a microscope stage (Axioskop FS-II, Zeiss), and continuously perfused with the Krebs' solution. Conventional tight-seal whole-cell recordings were obtained from neurons located in the superficial dorsal horn (lamina II) under visual control using infrared-differential interference contrast optics and a CCD video camera (IR-CCD 2741; Hamamatsu Photonics), as described previously [6]. The pipettes were filled with a solution of the following composition (in mM): K gluconate, 123; KCl, 14; Na gluconate, 2; EGTA, 1; HEPES, 10; and the pH of the solution was neutralized to 7.4 with KOH.

The currents were recorded in the voltage-clamp mode at a holding potential of -70 mV, using an Axopatch 200B patch-clamp amplifier (Axon Instruments). The data were sampled using a Digidata 1440 interface (Axon Instruments). A PCLAMP 10 (Axon Instruments) and Mini Analysis 6.0.3 (SynaptoSoft) were used to analyse the data.

2.5. Electrical stimulation-evoked EPSCs (eEPSCs) and spontaneously occurring miniature EPSCs (mEPSCs)

The external solution routinely contained strychnine (Sigma, 2–5 μM) and bicuculline

(Sigma, 10 μM). Electrical stimulation was applied using a glass pipette filled with 1M NaCl with its tip (diameter, ca 3 μm) placed at the dorsolateral margin of the spinal cord, 100–200 μm away from the recorded neuron. With a square pulse of 0.1 ms duration, stimulus intensity was adjusted so that an EPSC of similar amplitude was evoked in each experiment. Spontaneous mEPSCs were isolated by adding tetrodotoxin (TTX, 0.3 μM) to the external solution. Both eEPSCs and mEPSCs were abolished by 6-cyano-7-nitroquinoxaline-2,3-Dione (CNQX, 5 μM, data not shown), and thus identified as glutamatergic. To obtain a paired-pulse ratio (PPR), paired stimulations were applied (interstimuli interval of 50 ms).

2.6. Intrathecal injection of antisense oligodeoxynucleotide against the α2δ-1 subunit

For knockdown of calcium channel α2δ-1 expression in the spinal cord, antisense oligodeoxynucleotides (ODN) against α2δ-1 were intrathecally (IT) administered. The following ODNs were used (Gene Tools, Philomath, OR, USA): a morpholino antisense ODN against α2δ-1 (5'-CAGCAGGCAGCCAGCAGCCATCTCC-3') and a control nonsense ODN (5'-CCTCTTACCTCAGTTACAATTTATA-3'). Transfections were carried out using JetPEI (Polyplus-transfection SA). The animals were anesthetized with halothane, and the ODN was injected between the fifth and sixth lumbar vertebrae, by using a microsyringe with a 30-gauge needle, according to the procedure described by Hylden [7].

2.7. Quantitative reverse transcription-polymerase chain reaction

To assess the effects of sciatic nerve ligation and IT injection of antisense ODN on the expression of the α2δ-1 subunit mRNA, the superficial dorsal horn ipsilateral to the ligated sciatic nerve was excised from the L4–L5 spinal slices under a binocular stereoscopic microscope. Total RNA was isolated using an ISOGEN reagent

(Nippongene, Japan). Reverse transcription (RT) was performed with random hexamers by using a SuperScript cDNA synthesis kit (Invitrogen). Following RT, the cDNA was subjected to a real-time polymerase chain reaction (PCR) by using the Mastercycler ep realplex 2 system (Eppendorf, Hamburg, Germany). The cDNAs for α2δ-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified by using commercially available TaqMan gene expression assays (Mm00486607 and Mm99999915, respectively; Applied Biosystems). The relative expression of α2δ-1, normalized to that of GAPDH, was determined by using the comparative $2^{-\Delta\Delta Ct}$ method (Applied Biosystems).

The von Fray measurements were made every day from 2 days before surgery (sham operation or nerve ligation) through up to 10 days after surgery, until the animals were sacrificed for whole-cell recordings or mRNA analysis. The IT injections of antisense ODN were made three times (3, 5, 7 days after surgery). The whole-cell recordings or mRNA analysis were performed 10 days after surgery.

2.8. Statistical analysis

All data are presented as the mean ± SEM. Statistical analyses of the data were performed by one-way or two-way analysis of variance (ANOVA) followed by post hoc multiple comparison (Tukey test). The paired or unpaired Student's t -test was also used where appropriate. The level of significance was set at $p < 0.05$.

3. Results

3.1. Effects of PGB on eEPSCs in the spinal superficial dorsal horn of sham-operated mice

The bath application of PGB at a clinically relevant concentration of 100 μM inhibited the amplitude of eEPSCs reversibly in a sham-operated mouse (Fig. 1A, inset traces and the upper graph). This inhibition of eEPSC amplitude by PGB was accompanied by an increase in the PPR (Fig. 1A, the lower graph).

The same results were observed in 14 neurons of 6 sham-operated mice. In these neurons, PGB at 100 μM significantly inhibited the amplitude of eEPSCs to 83.4 ± 5.6% of the control (Fig. 2B-1, open circles, $p < 0.05$), and significantly increased the PPR value form 1.2 ± 0.5 to 2.3 ± 0.3 (Fig. 2B-2, open circles, $p < 0.05$).

3.2. Effects of PGB on mEPSCs in the spinal superficial dorsal horn of sham-operated mice

Figure 1B represents the effects of PGB at a concentration of 100 μM on mEPSCs in a sham-operated mouse. The cumulative histogram of the inter-event intervals of mEPSCs (Fig. 1B, the lower left graph) and that of the amplitude of mEPSCs (Fig.1B, the lower right graph) indicate that mEPSC frequency was decreased by PGB (Kormogoroff Smirnoff test, $p < 0.05$) without significant changes in mEPSC amplitude ($p > 0.05$).

The same results were observed in 15 neurons of 5 sham-operated mice. In these neurons, PGB decreased mEPSC frequency from 7.3 ± 0.9 Hz to 3.1 ± 0.5 Hz ($p < 0.05$). The amplitude of mEPSCs did not apparently change in response to PGB (from 18.1 ± 2.5 pA to 18.4 ± 3.3 pA, $p > 0.05$).

3.3. Alterations in the expression level of the $\alpha 2\delta$-1 subunit mRNA during peripheral nerve injury and the effects of IT injection of antisense ODN against $\alpha 2\delta$-1 subunit

The number of hindlimb withdrawal responses to 10 repetitive stimuli increased from 0 to 6.4±0.9 after the sciatic nerve ligation (n = 23, $p < 0.05$). After the IT injection of

antisense ODN, the withdrawal frequency was decreased to 1.4 ± 0.7 ($n = 9$, $p < 0.05$).

Real-time quantitative RT-PCR revealed that the expression of the calcium channel α2δ-1 subunit in the superficial dorsal horn changed in parallel to alterations in mechanical withdrawals (Fig. 2A). Sciatic nerve ligation-induced allodynia was accompanied with a significantly increased expression of the α2δ-1 subunit (Fig. 2A, filled bar). The IT antisense ODN-induced attenuation of allodynia was accompanied with a decreased expression of the α2δ□□ subunit (Fig. 2A, dotted bar).

Additionally, nonsense ODN was IT injected in sham-operated mice ($n = 3$), sciatic nerve-ligated mice ($n = 4$). The IT injection of nonsense ODN did not show any apparent effects on withdrawal responses or on α2δ-1 subunit expression.

3.4. Effects of PGB on eEPSCs during peripheral nerve injury and the effects of IT injection of antisense ODN against the α2δ-1 subunit

The inhibitory effects of bath application of PGB on eEPSCs were compared among sham-operated control mice, and sciatic nerve-ligated mice with or without IT injection of antisense ODN. In mice with sciatic nerve ligation, PGB inhibited eEPSC amplitude to 67.8 ± 7.2 % of the control (Fig. 2B-1, filled circles, $n = 15$, $p < 0.05$), which was significantly different from the effect observed in sham-operated mice ($p < 0.05$). Additionally, in sciatic nerve-ligated mice with IT injection of antisense ODN, PGB inhibited eEPSC amplitude to only 87.9 ± 4.3 % of the control, which was not a significant difference compared to before PGB (Fig. 2B-1, dotted diamond, $n = 11$, $p > 0.05$). The inhibition of eEPSC amplitude by PGB was accompanied with a significant increase in PPR as illustrated in Figure 2B-2.

3.5. Effects of PGB on eEPSCs in the superficial dorsal horn of sham-operated

syntaxin 1A knockout mice

The effects of PGB at a concentration of 100 μM on eEPSCs were compared between sham-operated syntaxin 1A knockout mice and sham-operated wild-type mice. As shown in Figure 3A, PGB reduced the eEPSC amplitude, and increased the PPR in a sham-operated syntaxin 1A knockout mouse.

In sham-operated syntaxin 1A knockout mice, PGB at 100μM inhibited eEPSC amplitude to 92.6 ± 2.9 % (n = 12) of the control (Fig. 3B-1 open circles). In sham-operated wild-type mice, PGB at 100 μM inhibited eEPSC amplitude to 83.1 ± 5.0 % (n = 11) of the control (Fig. 3B-1, filled circles). The inhibitory effect of PGB was significantly different between these two groups ($p < 0.05$). In both groups, inhibition of eEPSC amplitude was accompanied with an increase in PPR (Fig. 3B-2).

4. Discussion

4.1. PGB's presynaptic actions

The presently observed reduction of eEPSC amplitude with increased PPR is consistent with the previously suggested presynaptic site of action of gabapentinoids such as gabapentin and PGB [1-3, 8, 15]. Bayer et al. recorded glutamatergic eEPSCs from dorsal horn neurons, and found that gabapentin reversibly reduced eEPSC amplitude. They also found that gabapentin did not affect membrane currents elicited by glutamate application and concluded that gabapentin inhibits excitatory spinal neurotransmission via a presynaptic mechanism [1]. Biochemical measurement of glutamate amount has shown that gabapentin inhibited high K^+-induced glutamate release in hippocampal and neocortical slices [3]. Scintillation counting of radiolabeled glutamate in the trigeminal nucleus has revealed that gabapentin reduced the facilitation of glutamate release [8]. Additionally, our present observations could not exclude postsynaptic mechanisms of

action for gabapentin and PGB. For example, it has been reported that gabapentin differentially affects synaptic and extrasynaptic NMDA receptors expressed on postsynaptic neurons [11].

4.2. PGB acts as a ligand at the calcium channel α2δ subunit

Presynaptic inhibitory action of PGB was compared among sham-operated control mice, and sciatic nerve-ligated mice with or without IT injection of antisense ODN against α2δ-1 subunit.

The inhibitory actions of PGB were most prominent in sciatic nerve-ligated mice, which exhibited the highest expression of the α2δ-1. Intrathecal injection of antisense ODN reduced α2δ-1 expression and attenuated allodynia, and decreased presynaptic inhibitory action of PGB.

These observations supplement a growing body of evidence that gabapentin and PGB have a state-dependent action as a ligand at the calcium channel α2δ subunit [4, 9, 12, 16, 20].

4.3. PGB action could not be attributed solely to inhibition of calcium channels

The occurrence of mEPSCs recorded in the presence of TTX does not substantially depend on the influx of Ca^{2+} into the presynaptic terminals in the superficial dorsal horn [6]. Thus, the presently observed suppression of mEPSCs suggests that PGB's presynaptic inhibition in the superficial dorsal horn could not be attributed solely to the inhibition of Ca^{2+} influx.

Micheva et al., using cultured rat hippocampal neurons loaded with synaptic vesicle fluorescent dye probe FM464, found that both calcium-dependent and -independent dye release are reduced by PGB. They suggested that PGB inhibits synaptic vesicle release

independently of Ca^{2+} influx [10].

Cunningham et al. studied the effects of PGB and gabapentin on mEPSCs recorded in the entorhinal cortex *in vitro* [2]. Based on their observations that both drugs reduced mEPSC frequency without any apparent effects on their amplitude, it was concluded that these drugs presynaptically inhibit excitatory synaptic transmission through the mechanisms, as yet unidentified, occurring downstream of Ca^{2+} influx into the presynaptic terminals.

4.4. Direct or indirect interaction with syntaxin 1A might be one of the possible mechanisms for presynaptic action of PGB

Syntaxin 1A is a member of the SNARE proteins. It composes the presynaptic release machinery with SNAP-25, synaptobrevin, and other molecules, and plays an important role in the exocytotic release of neurotransmitters. Additionally, it has been reported that syntaxin 1A knockout mice exhibit the impairment of long-term potentiation in the hippocampal CA 1 region, although the basic synaptic transmission is normal [5]. More recently, we found that synaptic transmission in the spinal dorsal horn and nociceptive behavior were exaggerated following peripheral nerve injury in syntaxin 1A knockout mice [19]. In the present experiments, we have observed that the inhibitory effects of PGB were less noticeable in syntaxin 1A knock out mice. This might imply that syntaxin 1A is required for the full expression of presynaptic actions of PGB. Gabapentin and PGB can enter into cells via the L-amino acid transport system, and thus can act intracellularly on components of the vesicular exocytotic machinery [17, 18]. However, further studies are needed to investigate whether and how syntaxin 1A is involved in the analgesic action of PGB.

5. Conclusions

Extensive investigations have revealed the mechanisms underlying the actions of gabapentinoids such as bagapentin and PGB on neuropathic pain. The present study provided further evidence that PGB has presynatic actions in a state-dependent manner as a ligand at the calcium channel $\alpha 2\delta$ subunit. In addition, the results of experiments on syntaxin 1A knockout mice strengthen the evidence that PGB exerts its presynaptic inhibitory actions through the mechanisms occurring downstream of Ca^{2+} influx, and further suggest that direct and/or indirect interaction with exocytotic machinery which is composed of syntaxin, SNAP-25, synaptobrevin and other presynaptic proteins might be one of possible mechanisms for the presynatpic actions of PGB. However, identification of the mechanisms mediating interaction between PGB and exocytotic release machinery, if any exist, must await further study.

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Figure Legend

Figure 1: Representative recordings showing the inhibitory actions of PGB (100 μM) on eEPSCs (A) and mEPSCs (B) recorded from different two neurons in the superficial dorsal horn of sham-operated mice.

A) The upper graph shows the time course of inhibition of the first eEPSCs by PGB. The lower graph shows the time course of changes in PPR of the first over the second eEPSCs. Inset traces show examples of eEPSCs recorded at the time indicated by arrows (a) before, (c) during, and (c) after the application of PGB.

B) Traces show mEPSCs before (upper) and during (lower) the application of PGB. The lower left graph shows a cumulative histogram of the interevent intervals of mEPSCs (Kolmogorov-Smirnov test, $p < 0.05$). The lower right graph shows a cumulative histogram of the amplitude of mEPSCs ($p > 0.05$).

Figure 2: Expression level of the calcium channel α2δ-1 subunit and presynaptic actions of PGB in the superficial dorsal horn.

A) Changes in the expression level of the calcium channel α2δ-1 subunit in the spinal dorsal horn induced by peripheral nerve injury and IT injection of antisense ODN against the α2δ-1 subunit. The vertical axis indicates the expression level of the α2δ-1 subunit normalized to GAPDH. The horizontal axis indicates treatments that are: (1) sham-operated, (2) sciatic nerve ligated, and (3) sciatic nerve ligated and antisense ODN injected. Statistical comparison was made with one-way ANOVA followed by post hoc multiple comparison (Tukey test). *, $p < 0.05$.

B) Summary of inhibitory actions of PGB (100 μM) on eEPSCs amplitude (B-1) and PPR (B-2). Open circles represent sham-operated mice. Filled circles represent sciatic nerve-ligated mice. Dotted diamonds represents sciatic nerve-ligated and antisense ODN-injected mice. Statistical comparison was made with two-way ANOVA. *, $p < 0.05$.

Figure 3: Actions of PGB (100 μM) on eEPSCs in sham-operated syntaxin 1A KO mice.

A) Representative recordings showing the inhibitory actions of PGB (100 μM) on eEPSCs recorded from a sham-operated syntaxin 1A KO mouse. The upper graph shows the time course of inhibition of the first eEPSCs by PGB. The lower graph shows the time course of changes in PPR. Inset traces show example eEPSCs recorded at the time indicated by arrows (a) before, (c) during, and (c) after the application of PGB.

B) Summary of inhibitory actions of PGB (100 μM) on eEPSC amplitude (B-1) and PPR (B-2). Filled circles represent sham-operated WT mice. Open circles represent sham-operated syntaxin 1A KO mice. Statistical comparison was made with two-way ANOVA. *, $p < 0.05$.