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Effects of mirogabalin, a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels, on N-type calcium channel currents of rat dorsal root ganglion culture neurons

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Mirogabalin, which is a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels, is being developed for treating neuropathic pain including diabetic peripheral neuropathy and postherpetic neuralgia. Mirogabalin possesses unique $\alpha_2\delta$ subunit binding characteristics and has potent and long-lasting analgesic effects in neuropathic pain models. In the present study, we investigated the effects of mirogabalin on N-type calcium channel currents of the rat dorsal root ganglion (DRG) culture neurons using the whole-cell patch clamp technique. Small or medium DRG neurons were isolated from Sprague-Dawley rats and were incubated for 20 to 24 h with mirogabalin or pregabalin. The DRG neurons were depolarised from a holding potential of –40 mV to +40 mV in steps of 10 mV for 220 ms, and elicited N-type calcium channel currents were recorded. The N-type calcium channel currents were verified by sensitivity to ω-conotoxin GVIA, a selective N-type calcium channel blocker. Mirogabalin inhibited the calcium channel currents of rat DRG neurons at 50 μM, and pregabalin inhibited them at 200 μM. Mirogabalin and pregabalin showed significant differences in the peak current densities at depolarisation to –20 and –10 mV when compared with that shown by the vehicle control. In conclusion, mirogabalin inhibits N-type calcium channel currents in rat DRG culture neurons. The potent and long-lasting analgesic effects of mirogabalin are thought to be associated with its potent and selective binding to $\alpha_2\delta$-1 subunits and following functional inhibition of calcium channel currents.

1. Introduction

Gabapentinoids, such as pregabalin and gabapentin, are selective ligands for the $\alpha_2\delta$ subunit of voltage-gated calcium channels (Li et al. 2011; Alexander et al. 2015) and exert various pharmacological effects, including analgesic, anticonvulsant, and anxiolytic effects (Sills 2006; Dooley et al. 2007; Stahl et al. 2013). The $\alpha_2\delta$ subunits are multifunctional auxiliary proteins, which influence the trafficking, localisation, and biophysical properties of calcium channels (Dolphin 2012, 2013). Although the mechanism of action of gabapentinoids has not been fully elucidated, the inhibition of calcium channels and neurotransmitter release at the presynaptic endings of neurons is considered to be the predominant mechanism of action. The inhibition of neurotransmitter (e.g., glutamate, substance P, and calcitonin gene-related peptide) release attenuates neuronal hyperexcitability in the brain and spinal cord and contributes to various pharmacological effects as described above (Fehrenbacher et al. 2003; Sills 2006; Dooley et al. 2007; Taylor et al. 2007; Stahl et al. 2013).

Mirogabalin ([(1*R*,5*S*,6*S*)-6-(aminomethyl)-3-ethylbicyclo[3.2.0]hept-3-en-6-yl]acetic acid, Fig. 1) is a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels and is being developed for the treatment of neuropathic pain including diabetic peripheral neuropathy and postherpetic neuralgia. We have previously reported that mirogabalin shows potent and selective binding affinities for $\alpha_2\delta$ subunits and slower dissociation rates for the $\alpha_2\delta$-1 than for the $\alpha_2\delta$-2 subunit. Mirogabalin shows potent and long-lasting analgesic effects in rat models of neuropathic pain, and wide safety margins for side effects on the central nervous system. These pharmacological properties of mirogabalin are thought to be associated with its unique binding characteristics (Domon et al. 2018a, b).

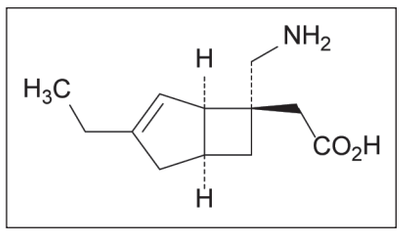

Fig. 1: Chemical structure of mirogabalin

In the present study, we investigated the effects of mirogabalin on high voltage-activated (HVA) calcium currents mediated by N-type calcium channels of rat dorsal ganglion (DRG) culture neurons.

2. Investigations, results and discussion

In total, 40 DRG neurons were evaluated for the HVA calcium current response in the present study. Furthermore, 15 neurons were sensitive to ω-conotoxin GVIA, a selective N-type calcium channel blocker (more than 70% block), while 25 neurons showed no such response. The ω-conotoxin GVIA responding neurons were classified as small or medium size DRG neurons (Scroggs and Fox 1992; Stemkowski et al. 2015); the size of the non-responding neurons was slightly larger, but the difference was not statistically significant (responding vs. non-responding neurons: 28.5±2.1 μm *vs*. 35.1±2.2 μm). Particularly, no correlation was found between the sensitivity to ω-conotoxin GVIA and the size of DRG neurons.

Figure 2 summarizes effects of mirogabalin and pregabalin on N-type calcium channel currents in 15 DRG neurons. The typical recordings of calcium currents are shown in the upper panel,

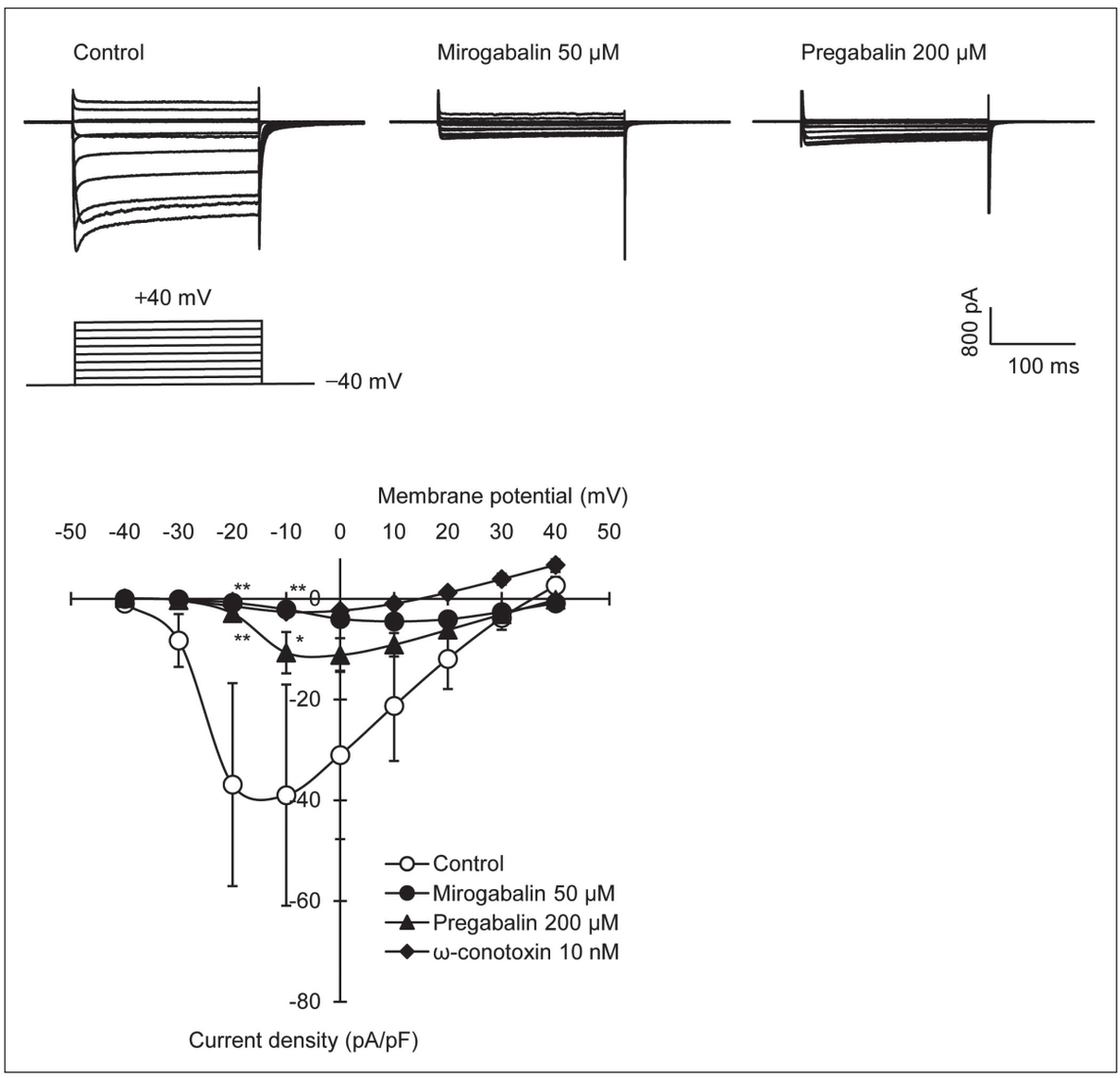

Fig. 2: Effects of mirogabalin and pregabalin on N-type calcium channel currents in rat DRG culture neurons. Upper panel: typical recordings of calcium currents obtained from a holding potential of −40 mV to +40 mV in +10 mV steps. Lower panel: I-V relationships of calcium currents established from a holding potential of −40 mV to +40 mV in steps of +10 mV. N-type calcium currents were recorded from rat DRG neurons cultured for 20 to 24 h in the absence or presence of mirogabalin (50 µM) or pregabalin (200 µM). Each value represents the mean ± standard error (N = 5). *$P < 0.05$, **$P < 0.01$: Significantly different from the control group (two-way ANOVA followed by a post hoc Bonferroni test). After I-V curve recordings of test compounds, the N-type calcium channel currents were verified by sensitivity to ω-conotoxin GVIA (10 nM for 5 min, N = 15).

and the current-voltage relationships (I-V curves) are shown in the lower panel. Mirogabalin (50 µM) and pregabalin (200 µM) inhibited the calcium currents in rat DRG neurons. The effects of mirogabalin and pregabalin in comparison with the control group were statistically significant at depolarisation to −20 and −10 mV. Although concentration-response relationships were not determined, the effect of mirogabalin at 50 µM was considered to be comparable to or more potent than that of pregabalin at 200 µM. The maximum peak current for the control and pregabalin groups was obtained at the −10 mV depolarising step, and that for the mirogabalin group shifted to a more depolarised value (+10 mV), indicating a delay in the activation.

We previously reported that mirogabalin had more potent and longer-lasting analgesic effects than pregabalin in neuropathic pain rat models, such as those involving partial sciatic nerve ligation, streptozotocin-induced diabetes, and spinal cord injury (Domon et al. 2018a, b). The binding affinity of mirogabalin to the $\alpha_2\delta$-1 subunit was greater than that of pregabalin. Further, mirogabalin had a longer half-life of dissociation from the $\alpha_2\delta$-1 subunit than from the $\alpha_2\delta$-2 subunit, in contrast to pregabalin (Domon et al. 2018a). The ratio of the effective concentrations of mirogabalin and pregabalin in the present study (50 µM vs. 200 µM) was parallel to the potency ratios of the K_d values associated with the binding affinity for the $\alpha_2\delta$-1 subunit (Domon et al. 2018a) and the effective doses in experimental animal models for neuropathic pain (Domon et al. 2018a, b).

In an experiment using a conventional protocol (comparison of observations before and after drug application for 10 min), mirogabalin at 50 µM and pregabalin at 200 µM tended to reduce the calcium current density. However, the decrease in the amplitude could be associated with an intrinsic run-down of the calcium channel since a similar change was observed in the control group (data not shown). Therefore, little or no effects on the calcium current density were observed following acute exposure (10 min) to mirogabalin and pregabalin, in contrast to the present study, which used chronic exposure (20–24 h). Gabapentin, a traditional $\alpha_2\delta$ ligand has been reported to show similar properties, and gabapentin is suggested to be an inhibitor of calcium channel trafficking, rather than a direct inhibitor of calcium channel currents (Hendrich et al. 2008; Heblich et al. 2008). Further, in co-culture of DRG and dorsal horn neurons, pregabalin has been reported to reduce capsaicin-induced increases in excitatory post synaptic

currents by chronic application (100 µM for 40–48 h), not by acute application (Hendrich et al. 2012). The multifunctional roles of $\alpha_2\delta$-1 subunits, such as regulating the functional membrane expression of voltage-gated calcium channels and modulating calcium currents, have been widely demonstrated (Davies et al. 2007; Dolphin 2012, 2013). The mechanism of action of gabapentinoids, such as pregabalin and gabapentin, involves both inhibition of calcium channel trafficking and direct effect on channel kinetics, which contribute to a reduction in calcium influx (Geisler et al. 2015; Kremer et al. 2016; Patel and Dickenson 2016).

In conclusion, mirogabalin inhibits N-type calcium channel currents in rat DRG culture neurons. The potent and long-lasting analgesic effects of mirogabalin are thought to be associated with its potent and selective binding for $\alpha_2\delta$-1 subunits and following functional inhibition of calcium channel currents.

3. Experimental

3.1. Chemicals

Mirogabalin (free-form of DS-5565, mirogabalin besylate) and pregabalin were synthesised by Daiichi Sankyo Co., Ltd. (Tokyo, Japan). The test compounds were dissolved in the culture medium at the final concentrations of 50 µM for mirogabalin and 200 µM for pregabalin. These concentrations were set as relatively higher levels than their therapeutic concentrations in clinical trials (Berry et al. 2005; Jansen et al. 2018) and animal experiments (Domon et al. 2018a), to obtain the apparent pharmacological effects under the limited experimental conditions (e.g., single concentration and minimum sample size). All other reagents were of analytical grade and obtained from conventional commercial sources.

3.2. Preparation of rat DRG neurons

All experimental procedures were performed in accordance with the French and European legislations for animal care, and the Guideline of the Institutional Animal Care and Use Committee of Daiichi Sankyo Co., Ltd.

Experiments were conducted using adult Sprague-Dawley rats (Elevage Janvier, France). The rats were euthanised by exsanguination after anaesthesia (pentobarbital 60 mg/kg, i.p.). The DRG neurons were quickly removed and immersed in ice-cold modified Hanks' balanced salt solution (HBSS) containing glucose 10 mM, HEPES 5 mM and 1% penicillin-streptomycin (pH 7.5). Enzymatic treatment of DRG neurons was first performed for 20 min with 0.2% collagenase and 0.5% dispase solution (2 mL) at 37 °C. Thereafter, the solution was removed and fresh collagenase-dispase solution (2 mL) was added for an additional 20 min. DRG neurons were then washed twice with pre-warmed modified HBSS. DRG neurons were taken in 1 mL culture medium containing Neurobasal A medium with 2% B27 supplement, 1% penicillin-streptomycin, and 2 mM l-glutamine. Single-cell suspensions were obtained by six passages through needles of decreasing diameters (21G, 23G, and 26G). Cells were seeded on glass coverslips coated with poly-d-lysine and laminin solution. After 2 h, the medium was replaced with fresh culture medium. DRG neurons were incubated overnight at 37 °C with 5% CO_2 before use.

3.3. Whole-cell patch-clamp recordings in DRG neurons

Recording set up: During the experiments, coverslips were continuously perfused at 2 mL/min with a solution containing tetraethylammonium bromide 150 mM, KCl 3 mM, $NaHCO_3$ 1 mM, $MgCl_2$ 1 mM, HEPES 10 mM, glucose 4 mM, $BaCl_2$ 1 mM, and tetrodotoxin 0.5 µM (pH set to 7.4 using KOH, osmolality set to 320 mOsm using sucrose). All the experiments were performed at room temperature. The recording pipette solution contained the following: Cs-aspartate 140 mM, EGTA 10 mM, $MgCl_2$ 2 mM, ATP-Tris 5 mM, GTP-Tris 1 mM, and HEPES 10 mM (pH adjusted to 7.2 using CsOH, osmolality adjusted to 310 mOsm using sucrose). Neurons were visualised using an inverted microscope (Olympus IX71) with a 40X objective. The diameters of all neurons with a round soma shape (typical shape for DRG neurons) were recorded. Whole-cell patch clamp experiments were performed in the voltage clamp mode using a software-controlled MultiClamp 700B amplifier in combination with a Digidata 1440A digitizer. Data were low-pass filtered at 2 kHz before being sampled at 10 kHz using Clampex 10.3 (all from Molecular Devices, LLC., CA, USA) on a personal computer. Patch pipettes were pulled from borosilicate glass capillaries with resistances of 2.5 to 4 MΩ.

Whole-cell configuration: A coverslip containing DRG neurons was placed on the patch-clamp setup. A patch-clamp pipette filled with intracellular solution was taken close to the membrane and negative pressure was applied to obtain a gigaseal resistance (resistance > 1 GΩ) between the pipette and DRG neuron membrane. Thereafter, negative pressure was applied to the pipette to break the membrane, which resulted in a whole-cell configuration, and emerging whole-cell capacitive currents were compensated using the circuitry of the patch clamp amplifier. Voltage clamping of neurons was performed at a holding potential of –40 mV.

Current-voltage relationship (I-V curve) recording: To record HVA calcium currents, DRG neurons were depolarised from the holding potential (–40 mV) to +40 mV in steps of +10 mV for 220 ms. Thereafter, the I-V curve was determined by measuring the peak current density (pA/pF) in response to each voltage step (mV). The I-V curve was always determined twice at 1-min intervals to ensure that the recorded I-V was stable. Test compounds were added to the culture medium for 20 to 24 h during the incubation prior to the recordings. I-V recording was performed after 3 min of recording stabilisation. To verify that the currents resulted from the opening of N-type calcium channels, ω-conotoxin GVIA (10 nM) was applied to the bath solution to block these currents. Data acceptance criteria were as follows: negative leakage current limit = −150 pA, Δleakage current limit = 50 pA, maximal series resistance (Rs) = 10 MΩ, and ΔRs < 50%.

3.4. Statistical analysis

Data are presented as the mean±standard error. Statistical analyses of the calcium current density were performed using a two-way ANOVA test followed by a post hoc Bonferroni test. Student's t-test was used to compare the size of the DRG neurons. Differences were considered significant when P was < 0.05. Prism 6 software (GraphPad Software Inc., San Diego, CA, USA) was used for these analyses.

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References

Alexander SPH, Catterall WA, Kelly E, Marrion N, Peters JA, Benson HE, Faccenda E, Pawson AJ, Sharman JL, Southan C, Davies JA, CGTP Collaborators (2015) The concise guide to pharmacology 2015/16: Voltage-gated ion channels. Br J Pharmacol 172: 5904 – 5941.

Berry D, Millington C (2005) Analysis of pregabalin at therapeutic concentrations in human plasma/serum by reversed-phase HPLC. Ther Drug Monit 27: 451 – 456.

Davies A, Hendrich J, Van Minh AT, Wratten J, Douglas L, Dolphin AC (2007) Functional biology of the $\alpha_2\delta$ subunits of voltage-gated calcium channels. Trends Pharmacol Sci 28: 220 – 228.

Dolphin AC (2012) Calcium channel auxiliary $\alpha_2\delta$ and β subunits: trafficking and one step beyond. Nat Rev Neurosci 13: 542 – 555.

Dolphin AC (2013) The $\alpha_2\delta$ subunits of voltage-gated calcium channels. Biochim Biophys Acta 1828: 1541 – 1549.

Domon Y, Arakawa N, Inoue T, Matsuda F, Takahashi M, Yamamura N, Kai K, Kitano Y (2018a) Binding characteristics and analgesic effects of mirogabalin, a novel ligand for the $\alpha_2\delta$ subunit of voltage-gated calcium channels. J Pharmacol Exp Ther 365: 573 – 582.

Domon Y, Kitano Y, Makino M (2018b) Analgesic effects of the novel $\alpha_2\delta$ ligand mirogabalin in a rat model of spinal cord injury. Pharmazie 73: 659 – 661.

Dooley DJ, Taylor CP, Donevan S, Feltner D (2007) Ca^{2+} channel $\alpha_2\delta$ ligands: novel modulators of neurotransmission. Trends Pharmacol Sci 28: 75 – 82.

Fehrenbacher JC, Taylor CP, and Vasko MR (2003) Pregabalin and gabapentin reduce release of substance P and CGRP from rat spinal tissues only after inflammation or activation of protein kinase C. Pain 105: 133 – 141.

Geisler S, Schöpf CL, Obermair GJ (2015) Emerging evidence for specific neuronal functions of auxiliary calcium channel $\alpha_2\delta$ subunits. Gen Physiol Biophys 34: 105 – 18.

Heblich F, Tran Van Minh A, Hendrich J, Watschinger K, Dolphin AC (2008) Time course and specificity of the pharmacological disruption of the trafficking of voltage-gated calcium channels by gabapentin. Channels (Austin) 2: 4 – 9.

Hendrich J, Van Minh AT, Heblich F, Nieto-Rostro M, Watschinger K, Striessnig J, Wratten J, Davies A, Dolphin AC (2008) Pharmacological disruption of calcium channel trafficking by the $\alpha_2\delta$ ligand gabapentin. Proc Natl Acad Sci USA 105: 3628 – 3633.

Hendrich J, Bauer CS, Dolphin AC (2012) Chronic pregabalin inhibits synaptic transmission between rat dorsal root ganglion and dorsal horn neurons in culture. Channels (Austin) 6: 124 – 132.

Jansen M, Warrington S, Dishy V, Ohwada S, Johnson L, Brown K, Ishizuka H (2018) A randomized, placebo-controlled, double-blind study of the safety, tolerability, pharmacokinetics, and pharmacodynamics of single and repeated doses of mirogabalin in healthy Asian volunteers. Clin Pharmacol Drug Dev 7: 661 – 669.

Kremer M, Salvat E, Muller A, Yalcin I, Barrot M (2016) Antidepressants and gabapentinoids in neuropathic pain: Mechanistic insights. Neuroscience 338: 183 – 206.

Li Z, Taylor CP, Weber M, Piechan J, Prior F, Bian F, Cui M, Hoffman D, Donevan S (2011) Pregabalin is a potent and selective ligand for $\alpha_2\delta$-1 and $\alpha_2\delta$-2 calcium channel subunits. Eur J Pharmacol 667: 80 – 90.

Patel R, Dickenson AH (2016) Mechanisms of the gabapentinoids and $\alpha_2\delta$-1 calcium channel subunit in neuropathic pain. Pharmacol Res Perspect 4: e00205.

Scroggs RS, Fox AP (1992) Calcium current variation between acutely isolated adult rat dorsal root ganglion neurons of different size. J Physiol 445: 639 – 658.

Sills GJ (2006) The mechanisms of action of gabapentin and pregabalin. Curr Opin Pharmacol 6: 108 – 113.

Stahl SM, Porreca F, Taylor CP, Cheung R, Thorpe AJ, Clair A (2013) The diverse therapeutic actions of pregabalin: is a single mechanism responsible for several pharmacological activities? Trends Pharmacol Sci 34: 332 – 339.

Stemkowski PL, Noh MC, Chen Y, Smith PA (2015) Increased excitability of medium-sized dorsal root ganglion neurons by prolonged interleukin-1β exposure is K^+ channel dependent and reversible. J Physiol 593: 3739 – 3755.

Taylor CP, Angelotti T, and Fauman E (2007) Pharmacology and mechanism of action of pregabalin: the calcium channel $\alpha_2\delta$ (alpha2-delta) subunit as a target for antiepileptic drug discovery. Epilepsy Res 73: 137 – 150.