

Gabapentin Attenuates the Activation of Transient Receptor Potential A1 by Cinnamaldehyde

Sangsu Bang, Sungjae Yoo and Sun Wook Hwang*

Korea University Graduate School of Medicine, Seoul 136-705, Korea

ABSTRACT

Gabapentin is used as an effective drug for relieving pain, but the main mechanism is still unclear. Recently, voltage-gated Ca^{2+} channel subunits are suggested for the main target for the analgesic action of gabapentin. We wonder whether gabapentin directly modulates other specific ion channels peripherally expressed in the sensory neurons. To test this, we used a heterologous expression system in which the cell lines transiently expressed thermosensitive transient receptor potential ion channels (thermoTRPs) as well as the primary cultured mouse trigeminal neurons. The application of gabapentin reduced the increases in the intracellular Ca^{2+} level caused by TRPA1 activation in the heterologous expression system whereas the responses via actions of other thermoTRPs were not dramatically affected by the gabapentin treatment. Gabapentin also attenuated the TRPA1-mediated intracellular Ca^{2+} increases in the cultured trigeminal neurons. These findings suggest TRPA1 in the peripheral sensory neurons as a novel target for the analgesic of gabapentin.

Key words: gabapentin, TRPA1, trigeminal ganglia, pain, CHO-K1

INTRODUCTION

Gabapentin was known to be an effective analgesic drug for variety types of pain such as neuropathic pain, fibromyalgia and perioperative pain, etc. (Garry et al., 2005; Cheng and Chiou, 2006; Arnold et al., 2007; Dworkin et al., 2007; Gilron et al., 2007; Nishiyori et al., 2008). The mechanism of the analgesic action of gabapentin is still not clear but the recent reports proposed that gabapentin might act on several receptors and ion channels expressed in the dorsal root ganglia (DRG) or in

the spinal cord dorsal horn neurons (Luo et al., 2001). Some of the predicted targets of the gabapentin actions are as follows: GABA_B receptors, K_{ATP} channels, α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptors and voltage-gated Ca^{2+} channels (Taylor et al., 1999; Freiman et al., 2001; Sills, 2006). Many studies showed that gabapentin or its potent analog, pregabalin binds to accessory subunits of voltage-gated Ca^{2+} channels (Gee et al., 1996; Sills, 2006). Other studies showed that gabapentin reduced the plasma membrane trafficking of β 4a-bound $\text{Ca}_v2.1$ complexes (Mich and Horne, 2008). However, there is a debate whether gabapentin is able to affect gating of the voltage-gated Ca^{2+} channels in heterologous expression systems or the primary cultured DRG neurons (Lanneau et al., 2001; Jensen et al., 2002;

*To whom correspondence should be addressed.

TEL: 82-31-412-6710, FAX: 82-31-412-6729

e-mail: sunhwang@korea.ac.kr

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Canti et al., 2003; Dooly et al., 2006).

Temperature-sensitive transient receptor potential ion channels (thermoTRPs) have been discovered in the peripheral sensory organs (TRPV1-4, TRPM8 and TRPA1) (Dhaka et al., 2006). Among the thermoTRPs, TRPA1 channel is emerging as an important pain sensor that is able to detect environmental, inflammatory or physical stimuli. TRPA1 mediates cold pain and bradykinin-induced pain (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004). TRPA1-deficient mice showed lack of sensitivities to its chemical ligands and impaired inflammatory pain sensation (Bautista et al., 2006; Kwan et al., 2006). Industrial irritants including acrolein is detected by TRPA1, thereby generating pain (Bautista et al., 2006). Mechanical pain sensation also seems to involve TRPA1 activation (Petrus et al., 2007). Agonists of TRPA1 have been found in the components in natural plants, environmental irritants and inflammatory compounds (Macpherson et al., 2007; McNamara et al., 2007; Materazzi et al., 2008; Taylor-Clark et al., 2008). For example, isothiocyanates (the pungent component in horseradish and wasabi), allicin (a component of garlic), cinnamaldehyde (an active compound in cinnamon oil), tetrahydrocannabinol (the psychoactive compound in marijuana), acetaldehyde (an ethanol metabolite), 4-hydroxy-2-nonenal (HNE) and prostaglandin J2 (an inflammatory mediator containing reactive α , β -unsaturated aldehydes), which elicit acute pain or neurogenic inflammation by activating TRPA1 expressed in the sensory neurons (Story et al., 2003; Bandell et al., 2004; Jordt et al., 2004; Bautista et al., 2005; Macpherson et al., 2005; Bang et al., 2007; Trevisani et al., 2007; Andersson et al., 2008; Andr e et al., 2008; Taylor-Clark et al., 2008). Because of such polymodal nature of TRPA1 in nociception, negative modulation of the TRPA1 activity using antagonists may be beneficial for suppressing diverse types of pain. AP18 and HC030031 are the latest examples for this strategy (McNamara et al., 2007; Petrus et al., 2007; Eid et al., 2008).

It has not been investigated whether gabapentin can modulate the activity of TRPA1. In this study, we found that gabapentin is able to attenuate the activation of TRPA1. The activities of other thermoTRPs in the sensory neurons were not dramatically affected by the gabapentin application, which

indicates that among the thermoTRPs, TRPA1 is the main target of gabapentin. These findings suggest TRPA1 may be a novel peripheral target for the analgesic action of gabapentin.

MATERIALS AND METHODS

Chinese Hamster ovarian (CHO)-K1 cell culture and Fluo-3 Ca^{2+} imaging

CHO-K1 cells were cultured in DMEM with 10% FBS and 1% penicillin/streptomycin. The CHO-K1 cells were transiently transfected with 0.5 μ g of individual thermoTRP plasmid DNA and 200 ng/well yellow fluorescent protein (YFP) cDNA in pCDNA6.2 by using Fugene HD (Roche Diagnostics Corp., Indianapolis, IN). For carrying out the Ca^{2+} imaging test, cells were plated on the glass cover slips and incubated for 24 h. The bath solution contained (in mM) 140 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES and adjusted to pH 7.4 with NaOH. For the voltage-gated channel experiments, 100 mM NaCl in the bath solution was replaced with 100 mM KCl. Cells were loaded with Fluo-3AM (5 μ M; at 37°C for 1 h) in the bath solution containing 0.02% pluronic acid (Invitrogen Corp., Carlsbad, CA). The levels of intracellular Ca^{2+} influx were measured with a confocal microscope (LSM5 Pascal, Carl Zeiss) and the images (488 nm excitation/514 nm emission) were collected every 3 s by using Carl Zeiss ratio tool software.

Trigeminal neuron culture and Fura-2 Ca^{2+} imaging

Adult mouse trigeminal ganglia were rapidly dissected out of ICR mice in cold phosphate-buffered saline solution and treated with 1.5 mg/ml collagenase/dispase (Roche Diagnostics Corp., Indianapolis, IN) in HBSS (Invitrogen Corp., Carlsbad, CA) containing 0.1% of penicillin/streptomycin at 37°C for 45 m and then treated with 0.25% trypsin (Invitrogen Corp., Carlsbad, CA) for 15 m. The collected tissue was mechanically triturated by using fire-polished Pasteur pipettes in DMEM supplemented with 5 mM HEPES at pH 7.4. Cells were plated onto poly-L-lysine-coated cover slips in DMEM/F12 containing 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, 5 ng/ml 2.5S NGF (Invitrogen Corp., Carlsbad, CA). Trigeminal neurons were

used for experiments after 48~72 h. The same bath solution described above was used. Cells were loaded with Fura-2AM (5 μ M; at 37°C for 1 h) in the bath solution containing 0.02% pluronic acid (Invitrogen Corp., Carlsbad, CA). Ca^{2+} imaging was carried out with a fluorescence microscope system (IX71, Olympus) and images (340 nm and 380 nm rational analysis) were collected every 3 s with MetaFlour 7.1 (Molecular devices, Downingtown, PA) ratio tool software. Neuronal cells were identified on the basis of KCl activity.

Compounds

All chemicals were purchased from Sigma-Aldrich unless otherwise described. The test solutions were made by dilution of the solutions which were dissolved in water, DMSO or ethanol before using.

Statistics

Data were analysed using the two-tailed Student's t-test and shown as means \pm S.E.M.

RESULTS

Gabapentin suppresses TRPA1 activity in the heterologous expression system

To test if the intracellular Ca^{2+} increase via TRPA1 activation was affected by the gabapentin treatment, we first used the CHO-K1 cell lines transiently expressing mTRPA1. The YFP-mediated fluorescence was normally detected in the majority of the CHO-K1 cells that were co-transfected with mTRPA1 cDNA and YFP cDNA (~80% of the total cells showed yellow fluorescence, Fig. 1A). Increases in the intracellular Ca^{2+} levels of the TRPA1-expressing CHO-K1 cells were shown in response to the cinnamaldehyde application to the bath in the Fluo-3 Ca^{2+} imaging experiments (Fig. 1B, C). The Ca^{2+} influx responses upon cinnamaldehyde was completely inhibited by the co-application of 3 mM gabapentin (n=21). Micromolar gabapentin showed a relatively weak inhibitory effect on TRPA1-mediated Ca^{2+} influx responses to cinnamaldehyde (n=47) compared to gabapentin at the millimolar concentration (Fig. 1B, C). The dose-response curve of the inhibitory effect of gabapentin on TRPA1 activation was also obtained from Fluo-3 Ca^{2+} ima-

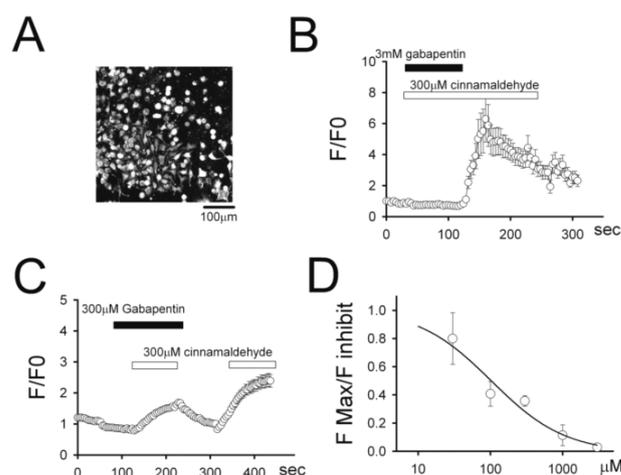


Fig. 1. Gabapentin suppresses TRPA1-mediated Ca^{2+} influx in CHO-K1 cells. (A) Transient expression of YFP in CHO-K1 cells 24 h after co-transfection of YFP and mTRPA1 cDNAs. YFP fluorescence was detected in approximately 80% of the transfected CHO-K1 cells when measured with the confocal microscope (scale bar=100 μ m). (B) 3 mM gabapentin completely blocked increases in the intracellular Ca^{2+} level in response to 300 μ M cinnamaldehyde in the mTRPA1-transfected CHO-K1 cells (n=21) in the Fluo-3 Ca^{2+} imaging experiments. Intracellular Ca^{2+} levels are displayed as mean \pm S.E.M. (C) The effect of 300 μ M gabapentin on the increases in the intracellular Ca^{2+} level in response to 300 μ M cinnamaldehyde in the mTRPA1-transfected CHO-K1 cells (n=38). (D) A dose-response curve of the inhibitory action of gabapentin on TRPA1 activation by 300 μ M cinnamaldehyde in Fluo-3 Ca^{2+} imaging experiments (IC₅₀=102.8 μ M, n=21~68). The curve was obtained by dividing the peak fluorescence level in response to cinnamaldehyde (F max) by the difference between the peak and the fluorescence level during the gabapentin co-application (F inhibit) and by fitting those data with Hill equation.

ging experiments using the TRPA1-expressing CHO-K1 cells (IC₅₀: 102.8 μ M, Fig. 1D). The curve indicates that gabapentin effectively suppressed the cinnamaldehyde-evoked Ca^{2+} influx responses in the TRPA1-cells throughout micromolar and millimolar ranges. These data suggest that TRPA1 activity in response to cinnamaldehyde is negatively modulated by the gabapentin treatment.

Sensitivities of thermoTRPs to gabapentin

To determine whether gabapentin also acts on other TRP channels, we examined changes in the Ca^{2+} influx of the CHO-K1 cells expressing individual thermoTRPs, TRPV1, TRPV2, TRPV3 and TRPV4. Values of the normalized inhibition were obtained by dividing the value of the Fluo-3 fluorescence increase in response to gabapentin plus

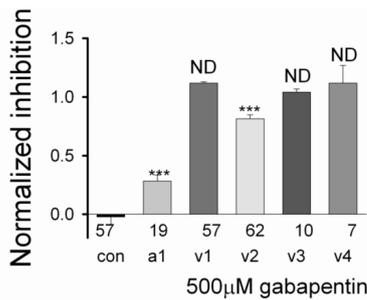


Fig. 2. The specificity of the action of gabapentin on the thermoTRP activity. The ratio of the intracellular Ca^{2+} levels upon application of specific agonists for individual thermoTRPs compared with those upon co-application of the agonists with gabapentin. TRPA1 showed dramatic suppression by the co-application of gabapentin in its activation ($p=0.0001$ for TRPA1; $p=0.78$ for TRPV1; $p=0.00001$ for TRPV2; $p=0.99$ for TRPV3; $p=0.37$ for TRPV4). Specific agonists used in this experiments were as follows: $0.2 \mu\text{M}$ capsaicin for TRPV1; $300 \mu\text{M}$ 2-APB (or $100 \mu\text{M}$ probenecid) for TRPV2; 4 mM camphor for TRPV3; $10 \mu\text{M}$ 4 α -phorbol 12, 13- didecanoate for TRPV4. Values of the normalized inhibition were obtained by dividing the value of the Fluo-3 fluorescence increase in response to gabapentin plus an agonist by that in response to the agonist alone. The control (con) value was obtained by dividing the value of the Fluo-3 fluorescence increase in response to gabapentin alone by that under the bath solution. As a result, the basal Ca^{2+} fluorescence level was not significantly affected by the gabapentin treatment. Con: control, A1: TRPA1, V1: TRPV1, V2: TRPV2, V3: TRPV3, V4: TRPV4, ND: not detected.

an agonist by that in response to the agonist alone. The activity of TRPA1 was dramatically blocked by gabapentin (Fig. 2, $n=19$, 72% suppression, $p=0.0001$) while TRPV1-, TRPV3- or TRPV4-expressing cells were not affected in their responses to specific activators by the co-application of gabapentin ($n=57$, 10, and 7 respectively). TRPV2-mediated Ca^{2+} influx was significantly suppressed by the co-application of gabapentin, but the effect was not comparable to the suppression of the TRPA1 responses (Fig. 2, $n=62$, 16% suppression, $p=0.00001$).

Gabapentin suppresses TRPA1 activity in the cultured sensory neurons

We also tested whether native TRPA1 responses in the cultured trigeminal neuron from mice were also suppressed by the gabapentin treatment in the Fura-2 Ca^{2+} imaging experiments. The TRPA1-positive trigeminal neurons were pharmacologically identified using cinnamaldehyde. We checked the healthiness and normal function of the sensory

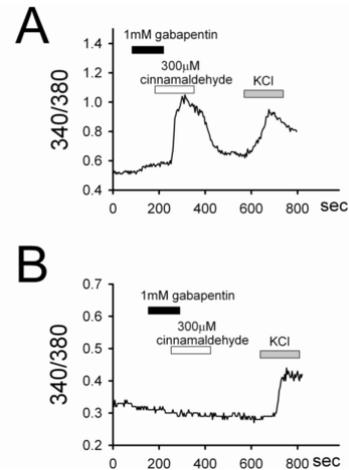


Fig. 3. Gabapentin suppresses TRPA1-mediated Ca^{2+} influx in the Fura-2 intracellular Ca^{2+} imaging experiments using the cultured mouse trigeminal neurons. (A) In the cinnamaldehyde-sensitive (TRPA1-positive) neurons, the increases of intracellular Ca^{2+} levels upon $300 \mu\text{M}$ cinnamaldehyde application were blunted by the gabapentin treatment ($n=8$). The neurons had normal Ca^{2+} influx responses via voltage-gated channels upon 100 mM KCl application. (B) Cinnamaldehyde-insensitive neurons did not elicit any change in the basal intracellular Ca^{2+} levels ($n=21$).

neurons by measuring Ca^{2+} -influx via KCl-induced depolarization after our pharmacological protocols at the end of the each experiments. A population of the neurons displayed cinnamaldehyde-evoked Ca^{2+} influx responses, which indicates that TRPA1 is expressed in a group of these neurons. The cinnamaldehyde responses were decreased by co-treatment of gabapentin ($n=9$, Fig. 3A) TRPA1-negative neurons (neurons insensitive to cinnamaldehyde, presumably the neurons that do not express TRPA1) did not show any change in the intracellular Ca^{2+} level upon the gabapentin treatment ($n=36$, Fig. 4B). These data demonstrated that gabapentin inhibits TRPA1-mediated Ca^{2+} influx in the sensory neurons.

DISCUSSION

In the present study, we showed that gabapentin suppressed TRPA1 activity using Ca^{2+} -imaging experiments in the CHO-K1 cell heterologous expression system. It is observed that gabapentin largely showed very little or no effect upon activity of other thermoTRPs. The inhibitory effect of gabapentin was reproduced in the experiments using TRPA1-

positive cultured trigeminal neurons.

We observed that TRPV2 activation by 2-APB, the TRPV2 agonist was slightly but significantly suppressed upon the gabapentin treatment although the effect was not comparable to the effect on TRPA1. Probenecid, the specific TRPV2 agonist did not elicit significant pain-related behaviors in mice under normal condition in our previous study (Bang et al., 2007). On the other hand, probenecid can induce pain-related behaviors in the inflammatory state (Bang et al., 2007). Although gabapentin does not appear efficacious enough to possibly lead to a change in TRPV2-related behavioral responses, it is conceivable that structural information of gabapentin or its analogs may be helpful to develop potent synthetic TRPV2 antagonists that negatively modulate TRPV2-related pain.

Little is known about the action mechanism of gabapentin and diverse targets for the drug have been proposed. The interaction between $\alpha_2\delta$ subunits of voltage-gated Ca^{2+} channel and the drugs explains the mechanism of action for their therapeutic effects for several clinical disorders, including epilepsy, pain from diabetic neuropathy, postherpetic neuralgia and fibromyalgia, and generalized anxiety disorder (Dooley et al., 2007). Gabapentin also inhibits the plasma membrane trafficking of β 4a-bound $\text{Ca}_v2.1$ complexes according to a recent report (Mich and Horne, 2008). Furthermore, pregabalin and gabapentin attenuated substance P-induced NF- κ B activation in human neuroblastoma and rat glioma cells system (Park et al., 2008). Other unknown molecules could be also involved in the analgesic mechanism regarding its broad spectrum of molecular targets and diverse therapeutic effects.

This study demonstrated that gabapentin inhibits TRPA1 activity. Because there are many types of TRPA1-dependent pain under normal and inflammatory condition, gabapentin may decrease pain sensation at least partly in a TRPA1-dependent manner, but this possibility remains to be answered in the future behavioral studies. It is also possible that the mechanical damage itself in surgeries and pre-surgical treated drugs including general anesthetics could increase gabapentin-sensitive pain and inflammation in the post surgical periods by a TRPA1-dependent mechanism (Matta JA et al.,

2008). Here, cinnamaldehyde, a pungent natural compound was used as a specific agonist to activate TRPA1. As mentioned above, variety of heterogeneous stimuli can activate TRPA1. Further study will clarify whether the inhibitory effect of gabapentin is agonist-specific. As well, it is also needed to determine whether gabapentin directly binds to TRPA1 protein or other cellular architectures coupled to TRPA1 in the plasma membrane. Gabapentin is a well known drug for relieving pain. The results in this study might provide useful information for explanation of a peripheral molecular mechanism of the analgesic action of gabapentin and also a novel TRPA1 modulating strategy.

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