Gabapentin Blocks L-Type and P/Q-Type Ca^{2+} Channels Involved in Depolarization-Stimulated Nitric Oxide Synthase Activity in Primary Cultures of Neurons from Mouse Cerebral Cortex

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Purpose: The effect of gabapentin [1-(aminomethyl)cyclohexane acetic acid] on Ca^{2+} channels involving the activation of nitric oxide synthase (NOS) was investigated in primary neuronal culture of mouse cerebral cortex.

Methods: The expression of $\alpha_2\delta$ subunits of Ca^{2+} channels was investigated by RT-PCR using specific primer sets. The K^+-evoked NOS activity was estimated by guanosine 3'5' cyclic monophosphate (cGMP) formation.

Results: mRNA for $\alpha_2\delta$ subunits of Ca^{2+} channels is found in these cells. Gabapentin blocked the K^+-evoked NOS activity estimated from cGMP formation in a concentration dependent manner. The increase in NOS activity by the K^+-stimulation was almost completely reversed by the combination of nifedipine, an L-type Ca^{2+} channel blocker, and ω-agatoxin VIA, a P/Q-type Ca^{2+} channel blocker. On the other hand, ω-conotoxin GVIA, an N-type Ca^{2+} channel blocker, was failed to reverse the increase in NOS activity by the K^+-stimulation, indicating that the activation of NOS by the depolarizing stimulation might be not mediated by N-type Ca^{2+} channel. Under the presence of nifedipine or ω-agatoxin IVA, gabapentin inhibited the increase in NOS activity concentration-dependently.

Conclusions: These results suggest that gabapentin inhibits depolarization-induced NOS activation in murine cortical neuronal culture *via* blockade of both P/Q-type and L-type Ca^{2+} channels.

KEY WORDS: gabapentin; ω-conotoxin GVIA; ω-agatoxin IVA; nitric oxide synthesis; cGMP; primary neuronal culture; mouse cerebral cortex; Ca^{2+} channels.

INTRODUCTION

Gabapentin is currently used with high efficacy in the treatment of neuropathic pain (1) and central nervous disorders, such as refractory epilepsy (2), even though gabapentin's mechanism of pharmacologic action remains elusive. However, the inhibition of Ca^{2+} channel function has been considered to be at least one of the activities of this compound. Recent researches have demonstrated that gabapentin binds to the $\alpha_2\delta$ subunits of voltage-gated Ca^{2+} channels with nanomolar affinity (3) and consequently inhibits Ca^{2+} currents in rat dorsal ganglia (4) and central nervous system neurons, such as neocortical neurons (5). Voltage-gated Ca^{2+} channels include low voltage-activated T-type channels, as well as high voltage-activated channels, such as L-type, P/Q-type, N-type, and R-type channels. Although the $\alpha_2\delta$ subunits are common in these Ca^{2+} channel subclasses, the precise role of the $\alpha_2\delta$ subunits in Ca^{2+} channel function is only partially understood, and little is known about the selectivity of gabapentin on each Ca^{2+} channel subclass. A few studies have shown that gabapentin inhibits all the Ca^{2+} currents through L-type, N-type, and P/Q-type Ca^{2+} channels in rat dorsal root ganglion cells (4). We have previously shown in cultures of mouse cerebral cortex neurons that L-type and P/Q-type Ca^{2+} channels are involved in the depolarization-induced activation of nitric oxide synthase (NOS), as demonstrated by the formation of guanosine 3′5′-cyclic monophosphate (cGMP; Refs. 6,7). Therefore, we compared the effect of gabapentin on L-type and P/Q-type Ca^{2+} channels involved in the depolarization-mediated NOS activation in primary cultures of mouse cerebral cortex neurons.

MATERIALS AND METHODS

Primary Neuronal Culture

Pregnant ddY mice (Shimizu Laboratory Supplies, Kyoto, Japan) were used. All animal procedures were carried out in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Animal Care and Use Committee of Kyoto Pharmaceutical University. Primary neuronal cultures were prepared from the cerebral cortex of 15-day-old fetal mice as described previously (7). In brief, trypsin-digested cells (1.45×10^6 cells/mL) were cultured on 24-well poly-L-lysine-coated plates (Falcon). The medium was replaced by freshly prepared Dulbecco's modified Eagle medium containing 15% fetal calf serum. Cells were treated on the third day with cytosine-β-D-arabinofuranoside (20 μM) to prevent the growth of non-neuronal cells. The culture medium was replaced every 4 days. Under these experimental conditions, contamination with astroglial cells was evaluated with an immunocytochemistry assay using antibodies against the glial fibrillary acidic protein, a specific marker for astroglial cells (7), and was determined to be less than 5% on the 14th day of culture.

Isolation of mRNA from Mouse Neuron Primary Culture and Reverse Transcriptase–Polymerase Chain Reaction (RT-PCR) Analysis

Total RNA was isolated from cultured neurons with Sepasol RNA I reagent (Nacalai Tesque, Kyoto, Japan) according to the manufacturer's instructions. Subsequently, poly(A)$^+$ RNA was isolated with Oligotex-dT30 (TaKaRa Biochemicals, Osaka, Japan), and $\alpha_2\delta$ subunit sequences were amplified by RT-PCR. The primers specific for mouse $\alpha_2\delta$-1 were 5′-AAC AGA TCT AAA GCC CTG GTG CGC C-3′ (forward primer) and 5′-ACC CAT GGA GAA GCT GGA TAA TAT CG-3′ (reverse primer) and amplify a complementary DNA (cDNA) fragment corresponding to nucleotide positions 504–901 (GenBank accession No. U73483). The primers specific for mouse $\alpha_2\delta$-2 were 5′-ATT GAC GGT GTG ATG CGG ATT TTT G-3′ (forward primer) and 5′-GAC ATC GTA CAG GTC AAT CTT CTT G-3′ (reverse primer) and amplify a cDNA fragment corresponding to nucleotide positions 693–1280 (GenBank accession No. AF247139). The primers specific for mouse $\alpha_2\delta$-3 were 5′-GTG GGG AGA

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TAA AAT CCA TCG CTG-3′ (forward primer) and 5′-GCT CTT TAA CTG GGA CAT CTG TGC-3′ (reverse primer) and amplify a cDNA fragment corresponding to nucleotide positions 149–1525 (GenBank accession No. NM_009785). PCR was performed with 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 90 s. The final cycle was followed by a single additional extension step of 72°C for 7 min.

Measurement of KCl-Evoked cGMP Formation in Mouse Neuron Primary Culture

NOS activity in cultured neurons was estimated from cGMP formation as described previously (7). The cGMP content in the supernatant was determined with the Amersham Biosciences cGMP Enzyme Immunoassay kit.

Statistical Analysis

Data were all analyzed with the SAS program (SAS/STAT, Version 6, fourth edition, 1990, SAS Institute Ins., Cary, NC, USA). Data were analyzed by one-way analysis of variance and then with Dunnett's test. The 50% inhibition concentration (IC_{50}) values were calculated by the least-squares linear regression method.

RESULTS AND DISCUSSION

RT-PCR Detection of mRNA for the $\alpha_2\delta$-1, -2, and -3 Subunits of Voltage-Gated Ca^{2+} Channels in Primary Cultures of Mouse Neurons

The $\alpha_2\delta$ subunit regulates many functional aspects of the Ca^{2+} channels, such as gating and regulating voltage-dependent kinetics (8). In addition to the regulation functions, the $\alpha_2\delta$-1 and $\alpha_2\delta$-2 subunits, but not the $\alpha_2\delta$-3 subunit, have been reported to be target sites for gabapentin binding (3,9). This binding affects neuronal excitability by modifying Ca^{2+} channel activity, which may be the mechanism by which gabapentin controls neuropathic pain (10,11). Therefore, we first used RT-PCR to investigate the expression of $\alpha_2\delta$ subunits in primary cultures of mouse cerebrocortical neurons. The RT-PCR results demonstrated that all three mouse $\alpha_2\delta$ mRNAs were expressed in neuronal cells (Fig. 1). These results indicate that gabapentin may regulate voltage-gated Ca^{2+} channel activity in mouse cortical neuronal cells.

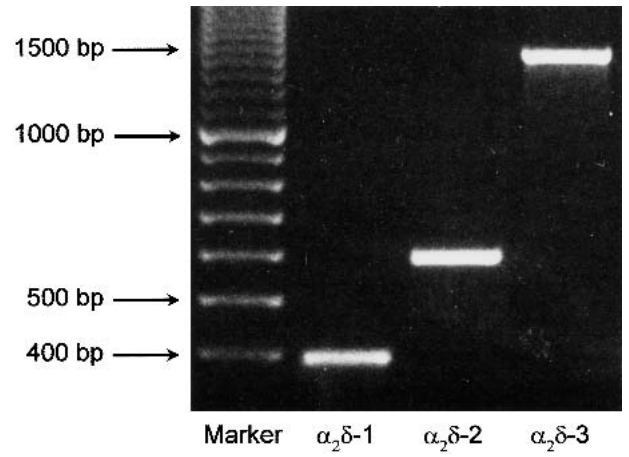

Fig. 1. Expression of $\alpha_2\delta$ subunits of Ca^{2+} channels in cultured neurons of mouse cerebral cortex. mRNA (0.5 μg) was reverse-transcribed and then subjected to polymerase chain reaction using primer pairs for $\alpha_2\delta$-1, $\alpha_2\delta$-2, and $\alpha_2\delta$-3. Expected size of polymerase chain reaction products is 398 bp, 588 bp, and 1377 bp for $\alpha_2\delta$-1, $\alpha_2\delta$-2, and $\alpha_2\delta$-3, respectively.

Effect of Gabapentin on KCl-Evoked cGMP Formation Is Mediated by Voltage-Gated Ca^{2+} Channels in Mouse Neuron Primary Culture

We previously reported that cGMP formation by mouse cortical neurons in primary culture is markedly reduced by NOS inhibitors, such as N^{ω}-nitro-L-arginine methyl ester (100 μM) and N^{ω}-monomethyl-L-arginine (100 μM), hemoglobin (10 μM) acting as a nitric oxide scavenger, calmodulin inhibitor W-7 (200 μM), or the removal of extracellular Ca^{2+}. These results indicate that cGMP formation reflects NOS activity (7). Gabapentin (10–300 μM) reversed KCl-stimulated NOS activity in a concentration-dependent manner, and a gabapentin concentration of 300 μM completely inhibited the activity (Fig. 2). In contrast, 300 μM gabapentin had no effect on NOS activation induced by the Ca^{2+} ionophore ionomycin (3 μM). Rates of cGMP production (mean ± SEM) were 0.538 ± 0.081 pmol/mg protein per min in the nontreated group (n = 6), 1.338 ± 0.115 pmol/mg protein per min in the ionomycin-treated control group (n = 6), and 1.418 ± 0.066 pmol/mg protein per min in the ionomycin + gabapentin–treated group (n = 6). Gabapentin inhibition of NOS activation was therefore specific for the KCl stimulation. In our previous report, we demonstrated that the activation of L-type and P/Q-type but not N-type Ca^{2+} channels mediated KCl-evoked NOS activity in primary cultures of neurons from the mouse cerebral cortex (7). Consistent with our earlier data, we found that the L-type Ca^{2+} channel blocker nifedipine (1 μM) and the P/Q-type Ca^{2+} channel blocker ω-agatoxin IVA (ω-Aga; 0.2 μM) reversed the KCl-evoked NOS activity by 60.0% and 37.1%, respectively, whereas ω-conotoxin GVIA, an N-type Ca^{2+} channel blocker, had no effect on the KCl response (data not shown). To identify the subclasses of Ca^{2+} channels affected by gabapentin, we examined

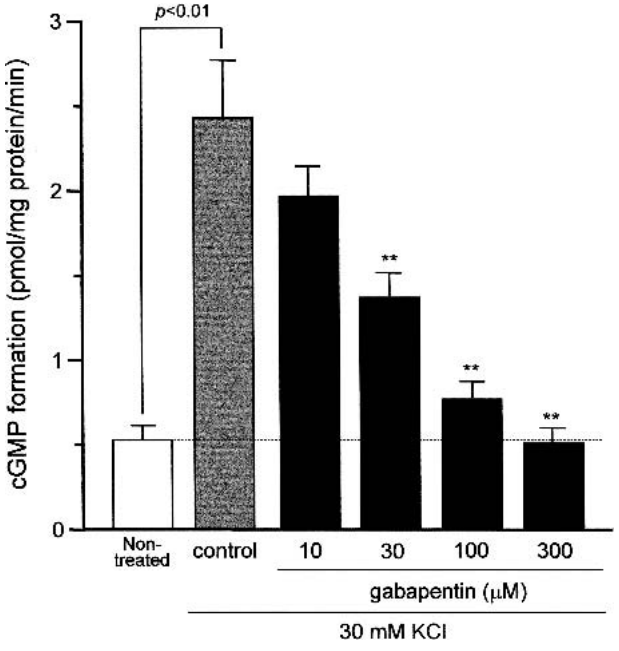

Fig. 2. Effect of gabapentin on the KCl-evoked NOS activity in primary neuronal culture. NOS activity was determined by cGMP formation. On the seventh day of culture, neuronal cells were preincubated at 37°C for 2 h with serum-free culture medium, followed by incubation for 15 min in the presence of 0.5 mM IBMX and various concentrations of gabapentin. Each column represents the mean ± SE of 6 experiments. **p < 0.01 as compared with KCl-stimulated control group.

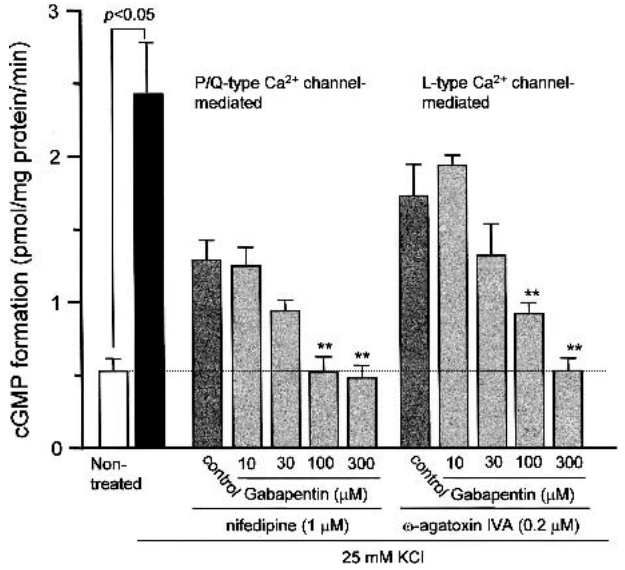

Fig. 3. Comparison of effects of gabapentin on KCl-induced cGMP formation mediated by P/Q-type and L-type Ca^{2+} channels in primary neuronal culture of mouse cerebral cortex. The KCl-evoked NOS activities mediated by L-type and P/Q-type Ca^{2+} channels were measured in the presence of 1 μM nifedipine and 0.2 μM ω-agatoxin IVA, respectively. Gabapentin was added to the incubation medium together with 0.5 mM IBMX and each Ca^{2+} channel blocker. Each column represents the mean ± SE of 6 experiments. **p < 0.01 as compared with nifedipine-treated or ω-agatoxin IVA-treated control group.

its effects on KCl-evoked NOS activities in the presence of the subclass-selective Ca^{2+} channel blockers. It is likely that the P/Q-type Ca^{2+} channel almost exclusively mediates the KCl-evoked NOS activity determined in the presence of nifedipine, whereas the L-type Ca^{2+} channel mediates nearly all of the activity in the presence of ω-Aga. As shown in Fig. 3, gabapentin (10-300 μM) inhibited to similar degrees the KCl responses determined in the presence of nifedipine and ω-Aga. The mean IC_{50} values were 35 μM (95% confidence limits, 21–52 μM) for the putative P/Q-type Ca^{2+} channel and 47 μM (95% confidence limits, 34–65 μM) for the L-type Ca^{2+} channel.

It is notable that the gabapentin concentrations inhibiting L-type and P/Q-type Ca^{2+} channel-mediated NOS activation were within the same range as the clinically relevant concentrations (10–100 μM; Ref. 12). Moreover, the IC_{50} values for gabapentin obtained from the present study are comparable with those reported by other investigators. The gabapentin IC_{50} values were 48 μM for the inhibition of KCl-evoked [^{3}H]-noradrenaline release (10) and 14 μM for the inhibition of KCl-induced elevation of $[Ca^{2+}]_i$ in rat neocortical synaptosomes (13). The nonselective blockade of Ca^{2+} channels by gabapentin has been reported by Sutton et al. (4), who showed in a whole-cell patch clamp study using rat dorsal root ganglion neurons that gabapentin inhibits N-type, L-type, and P/Q-type Ca^{2+} currents.

Ca^{2+} channels are implicated not only in pain signaling but also in the pathogenesis of ischemic brain damage. Several lines of evidence have demonstrated that ω-Aga inhibits formalin-induced nociceptive behaviors in rats (11). L-type Ca^{2+} channel blockers, including nifedipine, have been reported to produce a weak analgesia or to enhance the opioid analgesia (14). More recently, we have found that the nonselective Ca^{2+} channel blocker NS-7 reduces the infarct size in rat brains after middle cerebral artery occlusion (15) and causes a protective action in the *in vitro* hypoxic injury model using rat cerebrocortical slices (6). Like gabapentin, NS-7 blocks the P/Q-type and L-type Ca^{2+} channels equally to attenuate the KCl-evoked NOS activity in murine cortical neurons (7). In addition, our recent study, in which lactate dehydrogenase leakage in rat cerebrocortical slices after exposure to low oxygen and low-glucose medium for 45 min followed by reoxygenation for 6 h was used to determine hypoxic injury, found that gabapentin (100–1000 μM) significantly attenuated the tissue injury (unpublished observation). Taken together, these results suggest that the blockade of P/Q-type and L-type Ca^{2+} channels observed in the present study may account for the central actions of gabapentin, including its antiepileptic, anti-nociceptive, and neuroprotective actions.

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REFERENCES

1. A. Caraceni, E. Zecca, C. Martini, and F. De Conno. Gabapentin as an adjuvant to opioid analgesia for neuropathic cancer pain. *J. Pain Symptom Manage.* **17**:441–445 (1999).
2. R. E. Ramsay. Clinical efficacy and safety of gabapentin. *Neurology* **44**:S23–S30 (1994).
3. N. S. Gee, J. P. Brown, V. U. Dissanayake, J. Offord, R. Thurlow, and G. N. Woodruff. The novel anticonvulsant drug, gabapentin (Neurontin®), binds to the $\alpha_2\delta$ subunit of a calcium channel. *J. Biol. Chem.* **271**:5768–5776 (1996).
4. K. G. Sutton, D. J. Martin, R. D. Pinnock, K. Lee, and R. H. Scott. Gabapentin inhibits high-threshold calcium channel currents in cultured rat dorsal root ganglion neurons. *Br. J. Pharmacol.* **135**:257–265 (2002).
5. A. Stefani, F. Spadoni, and G. Bernardi. Gabapentin inhibits calcium currents in isolated rat brain neurons. *Neuropharmacology* **37**:83–91 (1998).
6. M. Oka, Y. Itoh, and Y. Ukai. Involvement of Na^+ and Ca^{2+} channel activation and resultant nitric oxide synthesis in glutamate-mediated hypoxic injury in rat cerebrocortical slices. *Life Sci.* **67**:2331–2343 (2000).
7. M. Oka, Y. Itoh, Y. Ukai, and K. Kimura. Blockade by NS-7, a neuroprotective compound, of both L-type and P/Q-type Ca^{2+} channels involving depolarization-stimulated nitric oxide synthase activity in primary neuronal culture. *J. Neurochem.* **72**: 1315–1322 (1999).
8. N. Qin, R. Olcese, E. Stefani, and L. Birnbamer. Modulation of human neuronal $\alpha_2\delta$ subunit. *Am. J. Physiol.* **274**:C1324–C1331 (1998).
9. M. Wang, J. Offord, D. L. Oxender, and T. Z. Su. Structural requirement of the calcium channel subunit $\alpha_2\delta$ for gabapentin binding. *Biochem. J.* **342**:313–320 (1999).
10. K. Fink, W. Meder, D. J. Dooley, and M. Gothert. M. Inhibition of neuronal Ca^{2+} influx by gabapentin and subsequent reduction of neurotransmitter release from rat neocortical slices. *Br. J. Pharmacol.* **130**:900–906 (2000).
11. A. B. Malmberg and T. L. Yaksh. Voltage-sensitive calcium channels in spinal nociceptive processing: blockade of N- and P-type channels inhibits formalin-induced nociception. *J. Neurosci.* **14**: 4882–4890 (1994).
12. K. Fink, D. J. Dooley, W. P. Meder, N. Suman-Chauhan, S. Duffy, H. Clusmann, and M. Gothert. Inhibition of neuronal Ca^{2+} influx by gabapentin and pregabalin in the human neocortex. *Neuropharmacology* **42**:229–236 (2002).
13. W. P. Meder and D. J. Dooley. Modulation, of K^+-induced synaptosomal calcium influx by gabapentin. *Brain Res.* **875**:157–159 (2000).
14. H. F. Miranda, D. Bustamante, K. Veronica, P. Teresa, S. Hernan, C. Paeile, E. Fernandez, and G. Pinardi. Anti-nociceptive effects of calcium channel blockers. *Eur. J. Pharmacol.* **217**:137–141 (1992).
15. Y. Takagaki, Y. Itoh, Y. Aoki, Y. Ukai, Y. Yoshikuni, and K. Kimura. Inhibition of ischemia-induced fodrin breakdown by a novel phenylpyrimidine derivative NS-7: an implication for its neuroprotective action in rats with middle cerebral artery occlusion. *J. Neurochem.* **68**:2507–2513 (1997).