Suppression of Formalin-Induced Nociception by Cilnidipine, a Voltage-Dependent Calcium Channel Blocker

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Cilnidipine is a 1,4-dihydropyridine-derived voltage-dependent calcium channel (VDCC) blocker and suppresses N-type VDCC currents in addition to L-type VDCC currents. An earlier investigation has suggested that intrathecally injected cilnidipine produces antinociception by blocking N-type VDCCs in mice. The present study using the rat formalin model examined antinociceptive effects of intrathecally and orally administered cilnidipine to elucidate a putative site of antinociception of cilnidipine, assess the efficacy of oral cilnidipine for pain relief, and clarify the mechanism(s) responsible for the antinociceptive effect of oral cilnidipine. Cilnidipine (whether intrathecal or oral) suppressed nociception in phases 1 and 2 of the formalin model. In addition, the potency of oral cilnidipine to suppress formalin-induced nociception in phase 2 was greater than that of oral gabapentin, a clinically available drug for treatment of neuropathic pain. Cilnidipine elicited antinociceptive effects without neurological side-effects including serpentine-like tail movement, whole body shaking, and allodynia. Such side-effects can be induced by higher doses of intrathecal ziconotide, a clinically available N-type VDCC blocker. In contrast, orally administered nifedipine, an L-type VDCC blocker, had no effect on either phase of formalin-induced nociception. These results suggest that cilnidipine acts on the spinal cord to produce antinociception and is efficacious for pain relief after oral administration with better safety profile than that of ziconotide. Furthermore, the failure of orally administered nifedipine to affect formalin-induced nociception raises the possibility that oral cilnidipine produces antinociception through, at least in part, spinal N-type VDCC blockade.

Key words cilnidipine; N-type voltage-dependent calcium channel; antinociception

Voltage-dependent calcium channels (VDCCs) facilitate calcium influx into cells, promoting neuronal functions in nervous systems. In particular, N-type VDCCs play a crucial role in nociceptive transmission. These channels are localized on presynaptic nerve terminals of small-diameter myelinated and unmyelinated afferents that synapse in laminae I and II of the spinal dorsal horn where they control neurotransmitter release. Ziconotide (also known as SNX-111), a synthetic form of ω-conotoxin MVIIA and an N-type VDCC blocker, exerts potent antinociceptive effects when injected intrathecally in animal models and clinical situations. Furthermore, N-type VDCC dysfunction resulting from N-type VDCC knockout in mice leads to higher nociceptive thresholds than in wild-type mice. N-type VDCCs are thus an important drug target for treatment of chronic pain. However, ziconotide must be intrathecally injected to be efficacious and can induce undesirable side-effects. Current efforts have been focused on development of orally efficacious N-type VDCC blockers with better safety profile.

Cilnidipine is a 1,4-dihydropyridine-derived VDCC blocker and suppresses N-type VDCC currents in addition to L-type VDCC currents. An earlier investigation has suggested that intrathecal cilnidipine produces antinociception by blocking N-type VDCCs in the mouse formalin model. Accumulating evidence has led us to believe that cilnidipine is the first member of a novel class of N-type VDCC blockers. However, it remains to be determined whether oral cilnidipine produces antinociception.

The present study examined the antinociceptive effects of intrathecal and oral cilnidipine in the rat formalin model to elucidate a putative site of antinociception of cilnidipine and the mechanism(s) responsible for the antinociceptive effect of oral cilnidipine as well as to assess the efficacy of oral cilnidipine for pain relief.

MATERIALS AND METHODS

Animals All experiments were performed according to protocols that were reviewed and approved by the Pharmaceutical Research Laboratories, Ajinomoto Co., Inc. (Kawasaki, Japan). Male Sprague-Dawley rats, weighing 300–380 g at the time of the experiments, were obtained from Charles River Japan (Yokohama, Japan) and were acclimatized to the laboratory environment for one week before starting the experiments. Animals were housed with a 12/12-h light cycle (0700—1900) and were allowed free access to food pellets and water.

Experimental Paradigm The rats were divided into 14 groups. The first four groups received intrathecal cilnidipine (control [0], 10, 30, or 100 ng). The other 10 received oral cilnidipine (0, 3, 10, or 30 mg/kg), gabapentin (0, 30, 100, or 300 mg/kg), or nifedipine (0 or 30 mg/kg). Intrathecal cilnidipine was injected 10 min before the formalin model, and oral cilnidipine, gabapentin, or nifedipine was administered 3 h before the formalin model.

Drug side-effects were assessed in animals with formalin-induced pain. In animals given intrathecal injection, serpentine-like tail movement and whole body shaking were assessed just before and after formalin injection, and the presence of allodynia was assessed just before formalin injection. In animals given oral administration, serpentine-like tail movement and whole body shaking were assessed 0.5, 1, 2, and 3 h after administration and after formalin injection, and the presence of allodynia was assessed 0.5, 1, 2, and 3 h after administration.

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**Catheter Implantation** For intrathecal drug injection, chronic intrathecal catheters were implanted in rats under halothane anesthesia according to a modification of the method described by Yaksh and Rudy.\(^3\) Through an incision in the atlanto-occipital membrane, a polyethylene catheter (PE-10) was inserted and advanced caudally to the rostral edge of the lumbar enlargement. The rostral segment of the catheter was subcutaneously tunneled to exit at the top of the head. After catheter implantation, rats were individually housed and monitored daily for signs of neurological dysfunction. Intrathecal injection was carried out at least 3 d after surgery. Only animals that displayed no post-surgical motor or sensory deficits were used in the experiments.

**Formalin Model** The formalin model was performed as previously described.\(^4\) Rats were placed in a Plexiglas box connected to a halothane vaporizer and allowed to breathe halothane (3%). After 2—3 min, there was a momentary loss of spontaneous movement with retention of deep spontaneous respiration, and the blink and pinnae reflexes. The animal was then removed quickly from the anesthesia box, and 50 µl of 5% formalin solution was subcutaneously injected into the dorsal surface of the left hind paw with a 30-gauge needle. Immediately thereafter, the rat was placed in a Plexiglas observation cylinder (30 cm in diameter and 35 cm in height), which was positioned in front of a mirror to allow unobstructed viewing of the hind paw. Within a maximum interval of 1—2 min, the animal recovered from the anesthesia and resumed spontaneous activity and normal motor function. This time point was defined as the start (0 min) of the formalin-model observation period. Noceptive behavior was quantified by periodical counting of spontaneous flinches of the injected paw. Animals were individually observed and the flinches were counted for 1-min periods which began at 1 min of a 60-min observation period of the formalin model and repeated at 5, 10, and every 5 min thereafter for the 60-min observation period. Two phases of spontaneous flinching behavior were observed as earlier described: phase 1 started at 0 min and lasted 5—6 min, and phase 2 began at 10 min.\(^3\) A drug’s effect was assessed from the cumulative number of flinches in each phase for each rat.

**Quantification of Side-Effects** Side-effects (serpentine-like tail movement, whole body shaking, and allodynia) were measured on a seven-point scale.\(^4\) The absence of serpentine-like tail movement and discernible whole body shaking resulted in respective scores of zero points. Mild serpentine-like tail movement or slight shaking or trembling of the body scored one point, moderate serpentine-like tail movement and whole body shaking scored two points, continuous serpentine-like tail movement and severe body shaking scored three points, and absence and presence of allodynia (assessed via a light brush-stroke to the flank) scored zero and one point, respectively. After the assessment, animals were sacrificed with an overdose of sodium pentobarbital.

**Drugs and Injections** For intrathecal injection, cilnidipine was dissolved in 10% dimethyl sulfoxide (DMSO) and 10, 30, or 100 ng was injected over approximately 15 s in a volume of 10 µl followed by saline to flush the catheter, which was attached by calibrated PE-90 tubing to a Hamilton glass syringe seated in a geared micro-injector. Conscious rats were gently restrained by swaddling in a towel during injection, and catheters were immediately replugged to prevent leakage of solutions. Control animals intrathecally received 10 µl of 10% DMSO only. For oral administration, cilnidipine and nifedipine were suspended in 0.5% solution of tragacanth gum (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and gabapentin was dissolved in distilled water. Cilnidipine (3, 10, or 30 mg/kg), gabapentin (30, 100, or 300 mg/kg), or nifedipine (30 mg/kg) was administered in a volume of 5 ml/kg by oral gavage. Control animals orally received 5 ml/kg of each vehicle only. Cilnidipine was synthesized by Ajinomoto Co., Inc. (Kawasaki, Japan). Gabapentin and nifedipine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

**Data Analysis and Statistics** The results (the number of flinches) are presented as mean±standard error (S.E.). ED\(_{50}\) (effective dose resulting in a 50% reduction of the control formalin response) and 95% confidence intervals (CI) were calculated by a least square linear regression method.\(^3,4\) Statistical analyses were performed with one-way analysis of variance (ANOVA) followed by Dunnett’s test for multiple comparisons with a single control, and with an unpaired t test for comparisons between two groups. p values less than 0.05 were considered statistically significant.

**RESULTS**

**Formalin Model** Subcutaneous injection of 5% formalin solution into the hind paw produced biphasic flinching behavior of the injected paw (Figs. 1A, 2A, 3A, 4A). No significant differences between control groups were found in the number of flinches in phase 1 or 2.

Cilnidipine, when injected intrathecally at 30 and 100 ng, produced significant suppression of the number of flinches in phase 1 (Fig. 1B). Furthermore, at 10, 30, and 100 ng, intrathecal cilnidipine dose-dependently produced significant suppression of the number of flinches in phase 2 (Fig. 1C). The ED\(_{50}\) values for cilnidipine in phases 1 and 2 were comparable (Table 1).

Oral cilnidipine (at 3, 10, 30 mg/kg) dose-dependently produced suppression of the number of flinches in both phases, and the ED\(_{50}\) values for phases 1 and 2 were also comparable (Figs. 2B, C and Table 1). In phase 1, the effect at 30 mg/kg was statistically significant (Fig. 2B). In phase 2, the effects at 10 and 30 mg/kg were statistically significant (Fig. 2C).

Oral gabapentin (at 30, 100, 300 mg/kg) tended to suppress the number of flinches in phase 1 by 30—35%, but the effects were not statistically significant (Fig. 3B, Table 1). These suppression rates prevented calculation of ED\(_{50}\) (i.e., the dose interval tested was out of the ED\(_{50}\) dose range). Oral gabapentin at 100 and 300 mg/kg resulted in significant suppression of the number of flinches in phase 2 (Fig. 3C). The ED\(_{50}\) value for gabapentin in phase 2 was approximately 12-fold higher than that for cilnidipine (Table 1).

Oral nifedipine, even at 30 mg/kg, had no effect on the number of flinches in both phases (Figs. 4B, C).

**Side-Effects** None of the drugs at doses used in the formalin model produced serpentine-like tail movement, whole body shaking, or allodynia.
DISCUSSION

The present study showed that intrathecally injected cilnidipine suppressed formalin-induced nociception in agreement with the earlier finding in the mouse formalin model, suggesting that the spinal cord is a putative site of antinociception of cilnidipine. Cilnidipine blocks N-type VDCCs and L-type VDCCs. A previous study reported that spinal L-type VDCC blockade produced moderate antinociception in the formalin model. Several lines of evidence, however, have suggested that L-type VDCCs play only a minimal role in spinal nociceptive transmission of formalin-induced pain. The present study has suggested that, in comparison with an L-type VDCC blocker nicardipine, intrathecal cilnidipine produces antinociception by blocking N-type VDCCs. In addition, Malmberg and Yaksh have reported that an L-type VDCC blocker nifedipine has no effect in the rat formalin model, when intrathecally injected even at 29 nmol. In the present study, intrathecal cilnidipine at 100 ng, the highest intrathecal dose examined in the formalin model, suppressed formalin-induced nociception. In other words, the suppression of formalin-induced nociception was produced by intrathecal cilnidipine at 0.20 nmol because molecular weight of cilnidipine is 492.5. When the dose unit was shown in nmol, the intrathecal dose of nifedipine (29 nmol) examined by Malmberg and Yaksh was approximately 150-fold higher than the highest intrathecal dose of cilnidipine (0.20 nmol) examined in the present study. In contrast, nifedipine produces levels of L-type VDCC blockade comparable to those seen for cilnidipine in vitro. On the basis of these findings, the intrathecal antinociception of cilnidipine in the present study can be attributed to spinal blockade of N-type VDCCs, which are responsible for neurotransmitter release.

The present study also revealed that both oral and intrathecal cilnidipine suppressed formalin-induced nociception in phases 1 and 2 to a comparable degree (60—70% in both phases at the highest dose examined). In the present study, furthermore, gabapentin, a clinically available drug for treatment of neuropathic pain, suppressed formalin-induced nociception in phase 2 with minimal effects on phase 1, in agreement with a previous finding. A comparison of cilnidipine- and gabapentin-produced antinociception in phase 2 (the persistent nociceptive phase) of the formalin model showed that cilnidipine had a greater potency on phase 2 nociception. These results suggest the efficacy of oral cilnidipine for pain relief.

In contrast to oral cilnidipine, oral nifedipine, which produces levels of L-type VDCC blockade comparable to those
seen for cilnidipine, had no effect on formalin-induced nociception under the present conditions. Given the previous and present results showing antinociceptive effects of oral and intrathecal cilnidipine in addition to the fact that an L-type VDCC blocker nicardipine had no effect on formalin-induced nociception, the failure of oral nifedipine to affect formalin-induced nociception suggests that oral cilnidipine produces antinociception, at least in part, through spinal N-type VDCC blockade. It should be noted that oral cilnidipine may also act on other sites to produce antinociception, since this drug is widely distributed throughout the body. Further studies are required to determine whether oral cilnidipine also exerts effects at the supraspinal and peripheral levels. In previous studies, intraperitoneal administration of L-type VDCC blockers had produced antinociception, the failure of oral nifedipine to affect formalin-induced nociception suggests that oral cilnidipine produces antinociception, at least in part, through spinal N-type VDCC blockade. It should be noted that oral cilnidipine may also act on other sites to produce antinociception, since this drug is widely distributed throughout the body. Further studies are required to determine whether oral cilnidipine also exerts effects at the supraspinal and peripheral levels. In previous studies, intraperitoneal administration of L-type VDCC blockers had produced antinociception, leading us to examine the antinociceptive effects of oral nifedipine in the present study. The reason for the difference in L-type VDCC blockade-produced antinociception between previous reports and the present study remains to be elucidated. The difference may depend on the measurement methods used, namely, behavior-scoring in the previous reports and flinch-counting in the present study.

Table 1. ED$_{50}$ Values and 95% Confidence Intervals (CI) for Intrathecally Injected (i.t.) and Orally Administered (p.o.) Drugs on Phases 1 and 2 in the Rat Formalin Model

<table>
<thead>
<tr>
<th>Drug</th>
<th>Phase 1 (0—9 min) ED$_{50}$ (95% CI)</th>
<th>Phase 2 (10—60 min) ED$_{50}$ (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cilnidipine i.t.</td>
<td>27 (19—39)</td>
<td>24 (17—35)</td>
</tr>
<tr>
<td>Cilnidipine p.o.</td>
<td>12 (7.8—18)</td>
<td>11 (7.8—16)</td>
</tr>
<tr>
<td>Gabapentin p.o.</td>
<td>&gt;300</td>
<td>130 (92—180)</td>
</tr>
</tbody>
</table>

In the present study, intrathecally or orally administered cilnidipine had no side-effects (i.e., failed to induce serpentine-like tail movement, whole body shaking, and allodynia). No neurological symptoms developed in mice which intracerebroventricularly received cilnidipine, whereas severe tremor followed intracerebroventricular injection of $\omega$-conotoxin GVIA, an N-type VDCC blocker. Despite blocking N-type VDCC currents, cilnidipine only partially displaces $\omega$-conotoxin GVIA binding which is totally displaced by $\omega$-conotoxin MVIIA. In contrast, ziconotide or $\omega$-conotoxin MVIIA, when injected intrathecally at higher doses that elicit marked antinociceptive effects, produces side-effects,
including serpentine-like tail movement, whole body shaking, or allodynia.\textsuperscript{4,5,33,52,53} For example, Chen \textit{et al.} showed that $\omega$-conotoxin MVIIA, when intrathecally injected at 0.1, 0.5, and 1.0 $\mu$g/kg, dose-dependently suppressed formalin-induced nociception in rats.\textsuperscript{54} Intrathecal $\omega$-conotoxin MVIIA at 1.0 $\mu$g/kg, however, caused whole body shaking and tail movement.\textsuperscript{55} These data suggest that the safety profile of cilnidipine is better than that of ziconotide. The manner by which cilnidipine blocked N-type VDCC currents was different from that by $\omega$-conotoxin MVIIA as noted above,\textsuperscript{56,57} and may contribute to the better safety profile of cilnidipine. Ziconotide and $\omega$-conotoxin MVIIA also share VDCC blockade characteristics, including relatively low selectivity for N-type VDCCs over P/Q-type VDCCs and no use-dependency in their blockade of N-type VDCCs.\textsuperscript{54—56} VDCC blockade by ziconotide or $\omega$-conotoxin MVIIA leads to deleterious effects on motor function and undesirable inhibitory effects on sympathetic functions such as orthostatic hypotension.\textsuperscript{56} In contrast, clinical and animal studies have shown that orthostatic hypotension is not induced by cilnidipine, suggesting that the safety profile of cilnidipine for autonomic reflex is also better than that of ziconotide.\textsuperscript{57,58} Differences in side-effect risks between cilnidipine and ziconotide should be further investigated.

Before starting the present study, it remained to be determined whether oral cilnidipine and nifedipine produced antinociceptive effects. Accordingly, we had no data on the dose at which each drug produced marked antinociceptive effect and the time-point when the antinociceptive effect of each drug peaked. Alternatively, we set the dose and the time-point for assessing the antinociceptive effect of each drug on the basis of their antihypertensive effects in spontaneously hypertensive rats (SHRs) because cilnidipine and nifedipine are clinically used for treatment of hypertension. Oral cilnidipine (3, 10, 30 mg/kg, \textit{p.o.}) produces levels of antihypertensive effects comparable to those seen for oral nifedipine (3, 10, 30 mg/kg, \textit{p.o.}) in SHRs.\textsuperscript{46} This finding thus suggests that cilnidipine and nifedipine exert comparable physiological effects (i.e., comparable antihypertensive effects) in the same dose range. In the present study, we thus examined antinociceptive effects of cilnidipine (3, 10, 30 mg/kg, \textit{p.o.}) and nifedipine (30 mg/kg, \textit{p.o.}). Because several lines of evidence suggest only a minimal role of L-type VDCCs in spinal nociceptive transmission of formalin-induced pain,\textsuperscript{4,30,36} we examined the antinociceptive effect of oral nifedipine at 30 mg/kg, \textit{p.o.}, equal to the highest oral dose of cilnidipine assessed in the present study. In addition, cilnidipine (3, 10, 30 mg/kg, \textit{p.o.}) and nifedipine (30 mg/kg, \textit{p.o.}) were orally administered at 3 h before the formalin model in the present study on the basis of two reasons. First, oral cilnidipine at each dose produces the maximum antihypertensive effect in SHRs at 3 h after administration.\textsuperscript{46} Second, a previous study has shown that the antihypertensive effect of oral nifedipine at 10 mg/kg peaks at 2 and 4 h after administration in SHRs.\textsuperscript{59} suggesting that the antihypertensive effect of oral nifedipine at “30 mg/kg” is also expected to peak at 2 and 4 h (including 3 h) after administration. We have thus considered peak of the antihypertensive effect of each drug to be possible peak of the antinoiceptive effect of each drug. On the basis of the design of experiments described above, oral cilnidipine (3, 10, 30 mg/kg, \textit{p.o.}) and nifedipine (30 mg/kg, \textit{p.o.}) were administered to rats with normal blood pressure (i.e., Sprague-Dawley rats) in the present study. Under this condition, cilnidipine (30 mg/kg, \textit{p.o.}) and nifedipine (30 mg/kg, \textit{p.o.}) decrease systemic blood pressure approximately by 20 mmHg.\textsuperscript{46} Although it remains to be examined whether cilnidipine-induced decrease in blood pressure influences antinoiceptive effects of this drug in the formalin model, taken together with the effect of oral nifedipine as above mentioned,\textsuperscript{59} the failure of oral nifedipine to affect formalin-induced nociception suggests that cilnidipine-induced decrease in blood pressure exerts little effect on antinoiceptive effects of cilnidipine under the present condition and also raises the possibility that oral cilnidipine produces antinoiceptive effects, at least in part, via spinal N-type VDCC blockade.

N-type VDCCs are an attractive therapeutic target for a variety of chronic and neuropathic pain conditions.\textsuperscript{10,15—19} Ziconotide produces antinoiception in tissue- and nerve-injured animal models and in clinical situations, whereas it has little effect on acute physiological pain. However, ziconotide must be intrathecally injected to be efficacious and can induce undesirable side-effects.\textsuperscript{3—10,31,36,60,61} Future directions include development of a novel class of N-type VDCC blockers with improved oral efficacy and better safety profile. Similar to ziconotide, cilnidipine is considered to be efficacious in tissue- and nerve-injured animal models with little effect on acute physiological pain, although systematic estimation of cilnidipine for antinoiception remains to be performed. In the future, structural optimization of cilnidipine to develop more selective N-type VDCC blockers is expected to be an important approach which provides promising new analgesics.

In conclusion, the present study suggests that cilnidipine acts on the spinal cord to produce antinoiception and is orally efficacious for pain relief with better safety profile than that of ziconotide. Furthermore, failure of oral nifedipine to affect formalin-induced nociception raises the possibility that oral cilnidipine produces antinoiceptive effect, at least in part, via spinal N-type VDCC blockade.

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