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Potentiation of Antinociceptive Effects of Morphine by Calcium-channel Blockers at the Level of the Spinal Cord

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Background: **Opioids inhibit voltage-dependent calcium-channel conductance, which is essential for the nervous system to be able to signal a painful event. Accordingly, interference with calcium-channel conductance may enhance opioid analgesia. The current study was designed to investigate the effects of calcium-channel blocking drugs on the antinociception of morphine at the level of the spinal cord.**

Methods: **Rats were chronically implanted with catheters in the lumbar intrathecal space. Tail-flick test was used to assess thermal nociception. Intrathecally administered drugs were morphine, calcium-channel blockers (verapamil, diltiazem, and nicardipine), or a combination of morphine and calcium-channel blocker.**

Results: **Intrathecal administration of morphine produced a significant dose-dependent antinociception in the tail-flick test. In contrast, intrathecal administration of calcium-channel blockers, verapamil, diltiazem, and nicardipine, did not show any antinociception at the employed doses. However, when intrathecally administered calcium-channel blockers, verapamil (50 μg), diltiazem (100 μg), or nicardipine (20 μg), were combined with ineffective (0.25, 0.5, 1, or 2 μg) or moderately effective (5 μg) doses of intrathecally administered morphine, significant antinociception was produced. These interactions were synergistic. There were no significant changes in MAP or HR after the intrathecal administration of 200 μg verapamil or 2 μg morphine combined with 50 μg verapamil.**

Conclusions: **The authors interpreted these results to indicate that calcium-channel blocking drugs synergistically potentiate the analgesic effects of morphine at the level of the spinal cord. Before these results can be translated into clinical use, however, adequate toxicity studies must be conducted to examine the effect of the perispinal administration of calcium-channel blocking drugs on spinal cord function. (Key Words: Analgesics, opioid: morphine. Anesthetic techniques: intrathecal; spinal. Pharmacology, calcium-channel blockers: diltiazem; nicardipine; verapamil.)**

MOVEMENT of calcium ions outside and within neurons is an important determinant in the functioning of the nervous system. Release of neurotransmitters is coupled with activation of voltage-dependent calcium conductance in synaptic terminal membranes of neurons. Nowycky *et al.*[1] reported the evidence for the coexistence of three distinct types of calcium channel in sensory neurons. These are the L, T and N channels. The L- and N-type calcium channels play a significant role in regulatory neurotransmitter release from neurons.

Because normal calcium movement is essential for normal sensory processing, a disruption of calcium ion movement, by interfering with normal sensory processing, should contribute to antinociception. It has been shown that intracerebroventricularly administered calcium can produce hyperalgesia in rodents,[2,3] and, conversely, that intracerebroventricular calcium chelators EGTA and EDTA, and the inorganic inhibitor of calcium cellular influx, lanthanum, produce antinociceptive effects that are antagonized by intracerebroventricular calcium.[4–6] Although it seems appropriate to assume that inhibitors of calcium cellular influx, including calcium-channel blockers, produce antinociception at the level of the spinal cord, some reports have indicated that intrathecally administered calcium produces antinociception.[7,8]

There is abundant experimental evidence of a close relationship between opioid effects and calcium transport through neuronal membranes in the central nervous system. Synaptosomal calcium content can be decreased by morphine.[9,10] It has also been demonstrated that morphine inhibits calcium ion influx through the receptor-operated calcium channel in neuronal cells.[11]

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The systemically administered L-type calcium-channel blockers verapamil and diltiazem have been shown to potentiate the antinociceptive effects of systemically or supraspinally administered morphine and other opiate receptor agonists.[12-17] Furthermore, it has been reported that calcium injections antagonize the analgesic effects of endogenous and exogenous opioids.[2,4,5,18] These observations indicate a functional relationship between opioids and calcium channels.

Opioids can act directly at the level of the spinal cord to produce analgesia. A proposed mechanism for opioid-mediated analgesia at the spinal level is thought to involve reducing presynaptic calcium ion influx, resulting in suppression of neurotransmitter release from primary afferents conveying nociceptive information.[9,19,20] This indicates that the antinociceptive effects of intrathecally administered opioids could be increased by intrathecal calcium-channel blockers.

Spinal opioid analgesia has played an important role in the management of acute and chronic pain. However, the side effects associated with intrathecal or epidural opioids are dose dependent, and respiratory depression, a major side effect of perispinal opioid administration, is life threatening and limits the clinical use of spinal opioids for pain management. If intrathecal calcium-channel blockers potentiate the analgesic effects of intrathecal opioids at doses that, alone, produce no, or only minimal, effects, then the combination of an opioid and calcium-channel blocker may be extremely useful for clinical pain management. The current study examined the effects of L-type calcium-channel blockers, verapamil, diltiazem, and nicardipine, on the antinociception of morphine at the level of the spinal cord.

Materials and Methods

Experiments were conducted in male Sprague-Dawley rats (250–350 g). Rats were housed singly in a temperature-controlled (21 ± 1° C) room with a 12-h light–dark cycle (lights on 7:00 AM to 7:00 PM) and given free access to food and water. The protocol of this study was approved by the Sapporo Medical College Animal Care and Use Committee.

Animal Preparation

Rats were anesthetized with halothane (2%) in oxygen. With the use of the modified method described by Bahar *et al.*,[21] an intrathecal catheter (PE-10) was inserted 15 mm cephalad into the lumbar subarachnoid space at the L4/5 intervertebrae with the tip of the catheter located near the lumbar enlargement of the spinal cord. The catheter was tunnelled subcutaneously to emerge at the neck. The volume of dead space of the intrathecal catheter was 8–10 μl. At least 6 days of postsurgical recovery were allowed before animals were used in experiments. In ten animals, a femoral arterial catheter was implanted for measurement of arterial blood pressure and heart rate. After experimental observations, each animal was killed by an overdose of halothane. Location of the distal end of the intrathecal catheter and the spread of injected material were determined by a postmortem intrathecal injection of 1% methylene blue (10 μl) followed by a flush of physiologic saline.

Nociceptive Test

To assess thermal nociceptive threshold, the tail-flick test was used. Standardized tail-flick testing was employed by monitoring latency to withdrawal from a heat source (a 50-W projection lamp bulb) focused on a distal segment of the tail. The location on the stimulated tail was systemically varied so that the same portion of the tail was not exposed repeatedly to the light source. The baseline tail-flick latency in the experiment was approximately 3.5 s, and a cutoff time of 10.0 s was employed to minimize damage to the skin of the tail. Animals were tested between 10:00 and 11:00 AM to control for diurnal fluctuations in opioid sensitivity.

Drugs and Injections

The drugs used were morphine hydrochloride (MW 375.85; Sankyo, Tokyo, Japan), verapamil hydrochloride (MW 491.07; Sigma, St. Louis, MO), diltiazem hydrochloride (MW 450.99; Tanabe, Osaka, Japan), nicardipine hydrochloride (MW 515.99; Yamanouchi, Tokyo, Japan), and naloxone hydrochloride (MW 363.84; Sankyo). Drugs were freshly dissolved in sterile physiologic saline in concentrations that allowed intrathecal injections in 10-μl volumes. All intrathecal injections were administered manually over 10 s and followed by a 10-μl flush of sterile physiologic saline to ensure that the drug reached the spinal cord. Control trials were conducted with intrathecally administered sterile physiologic saline. The current study was performed with 64 unanesthetized rats. Five to seven rats for each drug administration were used. There was at least 1 week between successive experiments with any rat after intrathecal administration of drug to avoid

possible sensitization to opioid effects, and each animal received, in total, two or three injections.

After determination of baseline tail-flick latencies, rats received intrathecal injections of morphine (0.5, 1, 2, 5, or 10 μg), verapamil (50, 100, or 200 μg), diltiazem (100, 200, or 500 μg), nicardipine (20, 50, or 100 μg), or morphine (0.25, 0.5, 1, 2, or 5 μg) combined with calcium-channel antagonists (50 μg verapamil, 100 μg diltiazem, or 20 μg nicardipine), in a random fashion. Tail-flick latencies were determined 10, 15, 30, 45, 60, 75, and 90 min after intrathecal drug administration. In the animals that were administered the combination of morphine and calcium-channel blocker, 200 μg/kg naloxone was administered intraperitoneally 91 min after administration of the combined drugs, and latencies were again evaluated 5 min after naloxone.

The solution *p*H of morphine, calcium-channel blockers, and morphine–calcium-channel blocker mixture in physiologic saline were analyzed by an ABL3 (Radiometer, Copenhagen, Denmark). The *p*H of a 10-μl solution of 2 μg morphine, 50 μg verapamil, 100 μg diltiazem, 20 μg nicardipine, 1 μg morphine/50 μg verapamil, 1 μg morphine/100 μg diltiazem, and 1 μg morphine/20 μg nicardipine were 6.34, 5.80, 5.37, 4.92, 5.72, 5.36, and 4.74, respectively.

Cardiovascular Measurement

To examine the cardiovascular effects of intrathecal calcium-channel blockers, arterial blood pressure, and HR were measured before and after drug administration in the animals in which an arterial catheter had been inserted.

Statistical Analysis

The response for the tail-flick test was calculated as the percent maximum possible effect (%MPE): %MPE = (postdrug latency − baseline latency)/(cutoff time − baseline latency) × 100. Dose responses were constructed and compared using measurement at a set time in each animal (15 min after administration). The effects of drugs on tail-flick latency were evaluated by ANOVA, followed by Student's *t* test. A *P* value < 0.05 was considered to be statistically significant. To assess the interaction between morphine and calcium-channel blockers on nociception, the ED_{50} values with 95% confidence intervals (CI) of morphine alone and morphine with calcium-channel blockers were calculated. If the CI of the morphine alone did not overlap with the CI of the morphine with calcium-channel blocker, a synergistic interaction was present.[22] The ED_{50} ratio (ED_{50} of morphine/ED_{50} of morphine with calcium-channel blocker) indicates an index of the extent of the synergistic interaction.

Results

Intrathecally administered morphine produced a significant prolongation of tail-flick latency in a dose-dependent manner (fig. 1). In contrast, intrathecally administered calcium-channel blockers, verapamil, diltiazem, and nicardipine, did not show any prolongation of tail-flick latency at the employed doses (fig. 1). Intrathecal physiologic saline, as a control, did not cause any changes in latency (data not shown).

Figure 2 demonstrates the effects of an ineffective dose (2 μg) or moderately effective dose (5 μg) of intrathecally administered morphine combined with 50 μg verapamil. In contrast to the ineffectiveness of 2 μg morphine alone, the combination of 2 μg morphine and 50 μg verapamil produced a significant prolongation of the latency ($P < 0.01$). Moreover, the moderately effective dose of intrathecal morphine (5 μg) combined with verapamil also significantly increased the latency when compared with 5 μg morphine alone ($P < 0.01$). The duration of this effect was also much greater. These potentiations were reversed by intraperitoneal naloxone. Figures 3 and 4 show the effects of the ineffective dose of intrathecally administered morphine (2 μg) combined with 100 μg diltiazem and

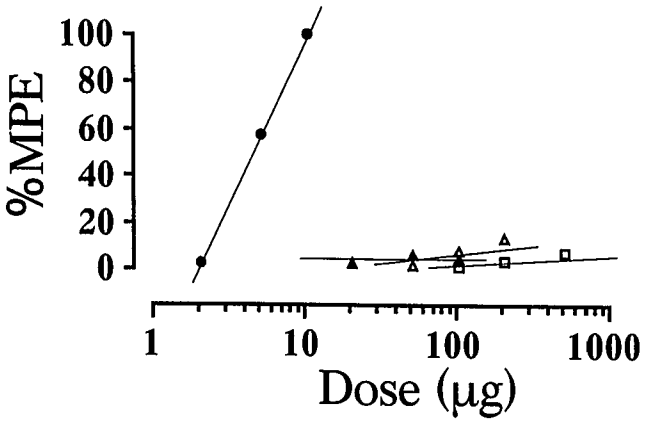

Fig. 1. Dose-response lines for morphine (filled circles), verapamil (open triangles), diltiazem (open squares), and nicardipine (filled triangles). The points represent the %MPE seen 15 min after intrathecal administration of drugs. The number of observations at each point was five to seven.

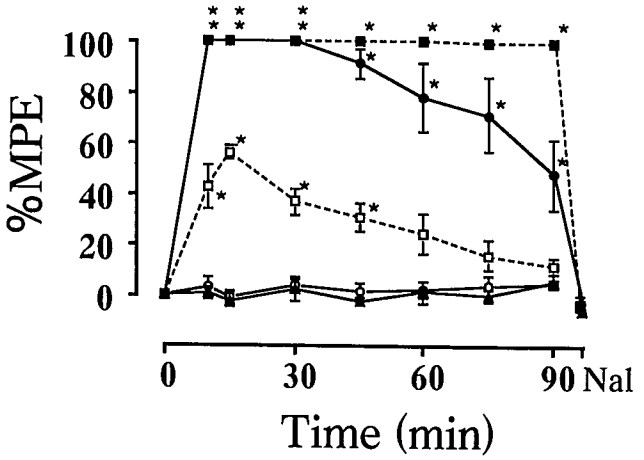

Fig. 2. Effects of the combination of morphine and verapamil on tail-flick latency. Although neither 2 μg morphine (open circles, n = 6) nor 50 μg verapamil (filled triangles, n = 5) produced any prolongation of tail-flick latency for 90 min, the combination of 2 μg morphine and 50 μg verapamil (filled circles, n = 6) produced significant prolongation. When a moderately effective dose of morphine (5 μg) (open squares, n = 5) was combined with 50 μg verapamil, significant prolongation of tail-flick latency was shown (filled squares, n = 7). The effects of the combinations were reversed by intraperitoneally administered naloxone (Nal; 200 μg/kg). *$P < 0.01$ compared with the baseline values of preadministration.

20 μg nicardipine, respectively, on the tail-flick latency. These combinations also produced significant antinociception ($P < 0.01$), and the effects were reversed by intraperitoneal naloxone.

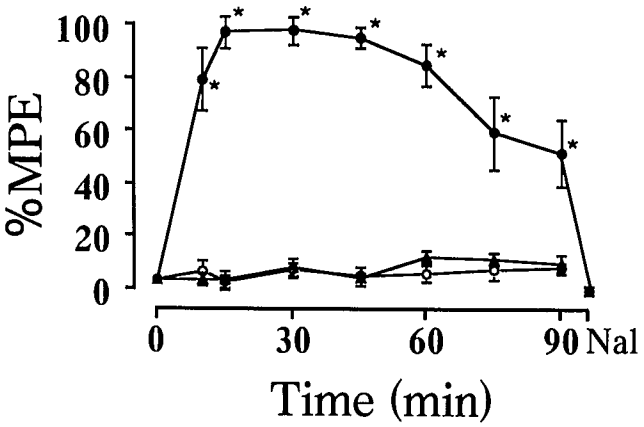

Fig. 3. Effects of 2 μg morphine (open circles, n = 6) combined with 100 μg diltiazem (filled triangles, n = 7) in the tail-flick test. The combination (filled circles, n = 7) produced significant prolongation, and the effect was reversed by intraperitoneal naloxone (Nal; 200 μg/kg). *$P < 0.01$ compared with the baseline values of preadministration.

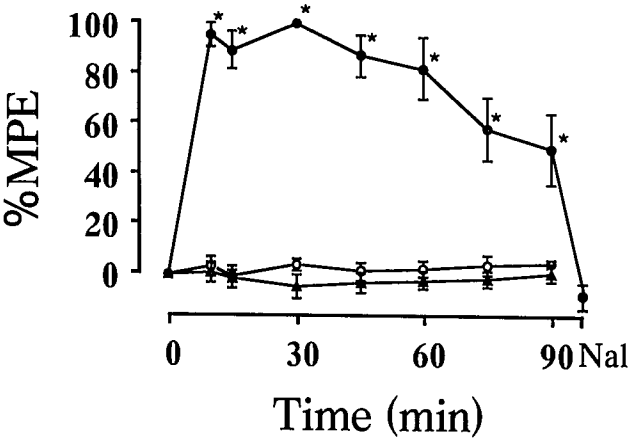

Fig. 4. Effects of 2 μg morphine (open circles, n = 6) combined with 20 μg nicardipine (filled triangles, n = 7) in the tail-flick test. The combination (filled circles, n = 6) produced significant prolongation, and the effect was reversed by intraperitoneal naloxone (Nal; 200 μg/kg). *$P < 0.01$ compared with the baseline values of preadministration.

In the systematic dose-response analysis, 50 μg verapamil, 100 μg diltiazem, and 20 μg nicardipine resulted in a significant leftward shift of the log dose-response curves for the curve for morphine alone (fig. 5). Confidence intervals of morphine alone and morphine combined with calcium-channel blockers did not overlap; therefore, these results indicate a potent synergistic interaction between morphine and calcium-channel blockers. The ED_{50} ratios were comparable between verapamil, diltiazem, and nicardipine (table 1).

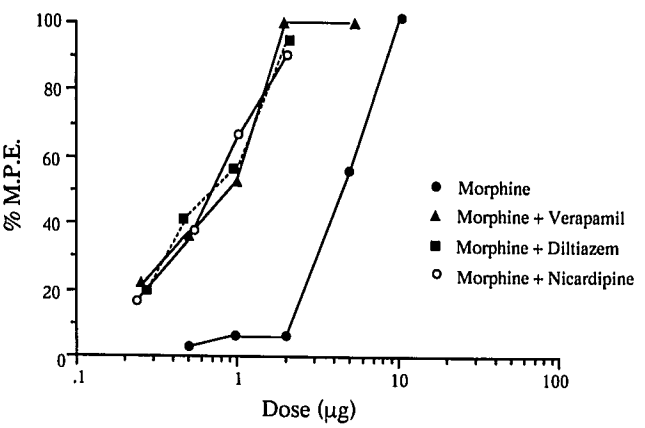

Fig. 5. The dose-response curves for morphine and morphine combined with calcium-channel blockers (verapamil, diltiazem, and nicardipine). The data points represent %MPE seen 15 min after intrathecal administration of drugs. The number of observations at each point was five to seven.

There were no significant changes in MAP or HR after the intrathecal administration of 200 μg verapamil or 2 μg morphine combined with 50 μg verapamil (data not shown).

Discussion

Several previous studies have indicated that systemically administered calcium-channel blockers potentiate the analgesic effects of systemically administered opioids.[13–15,17] The current study has demonstrated that the intrathecal administration of calcium-channel blockers, which alone exert no influence on tail-flick latency, significantly potentiate the analgesic effects of intrathecal administration of morphine in rats. This interaction is fully consistent with a synergistic interaction between the morphine and calcium-channel blockers on antinociception at the level of the spinal cord. This synergy was confirmed by the fact that the EC_{50}s with 95% CI for the morphine combined with calcium-channel blockers did not overlap the ED_{50} with 95% CI for the morphine alone.

Because influx of calcium ions into nerve endings is coupled with exocytotic release of neurotransmitters, the movement of calcium ions through calcium channels of neurons is an important determinant in the functioning of nervous systems. Experimental evidence from various sources has indicated that peripheral and central nervous system neurons possess several types of voltage-dependent calcium channels. The coexistence of three types of calcium channel (L, N, and T) in sensory neurons of the dorsal root ganglion has been designated.[1] The drugs we tested are selective for the L-type channel,[23,24] and, in fact, the L channels are defined by being sensitive to the dihydropyridines and, therefore, the data support involvement of L-type channels. In the tail-flick test, a behavioral thermal nociceptive assay, we demonstrated that intrathecally administered calcium-channel blockers alone (verapamil, diltiazem, and nicardipine) did not produce antinociception. Our failure to observe antinociception after pharmacologic blockade of L-type calcium channels indicates that those channels that are sensitive to dihydropyridines may not be directly involved in neurophysiologic responses that are important to the spinal processing of nociceptive information. This result is supported by the several previous reports in which systemically administered calcium-channel blockers did not change the reaction time to thermal stimulation.[17,25]

Table 1. ED_{50} Values for Intrathecal Morphine and Intrathecal Morphine Combined with Calcium Channel Blockers

	ED_{50} (CI) (μg)	ED_{50} Ratio
Morphine	5.1 (4.2–6.3)	—
Morphine + verapamil	0.88 (0.74–1.00)	5.8
Morphine + diltiazem	0.81 (0.61–1.00)	6.3
Morphine + nicardipine	0.83 (0.37–1.27)	6.1

CI = 95% confidence interval.

Inhibition of the voltage-dependent calcium conductance, and consequent lowering of calcium ions of the neurons by opioids, results in analgesia. Opioids block receptor-operated calcium channels in a naloxone-sensitive manner.[4] Analgesia may require the blockade of receptor-operated calcium channels, because calcium-channel blocker alone, which directly blocks voltage-dependent calcium channels, would produce insufficient analgesia, as seen in the results in this study. Morphine blocks the channels associated with the opioid receptor to reduce neurotransmitter release; therefore, additional direct blockade of voltage-dependent calcium channels by calcium-channel blockers would further reduce neurotransmitter release and, thus, potentiate analgesia.

Recently, some reports have demonstrated that the intrathecal calcium produces antinociception and potentiates intrathecal morphine antinociception. Proposed mechanisms involved the release of endogenous opioid or adenosine in the spinal cord. Welch *et al.*[8] have hypothesized that intrathecal calcium raises intracellular-free calcium in opioid-containing neurons, resulting in the release of endogenous opioids, which, in turn, decrease intracellular calcium and other second messengers in substance P-containing neurons. Furthermore, the study of Sawynock *et al.*[26] demonstrated that intrathecal calcium released adenosine from nerve terminals of small-diameter primary afferent neurons, and subsequent activation of adenosine receptors potentiated the action of morphine. Although there may appear to be conflict in that both calcium-channel blockers and calcium ions potentiate the antinociception of morphine, these effects are probably caused by quite different mechanisms; the direct inhibition of calcium ion influx and consequent decrease in neurotransmitter release *versus* the induction of releasing opioid or adenosine.

Spinal opioid analgesia for somatic nociceptive stimulation is mediated through μ or δ opioid receptor sub-

types.[27,28] Omote *et al.*[29] indicated that intrathecal morphine produced antinociception as a result of an interaction with δ, as well as μ, opioid receptors. It has been shown that systemic calcium-channel blockers augment the analgesic effects of μ and δ opioid agonists.[15,16] Therefore, it seems that the activator for μ and δ opioid receptors, morphine, interacts with intrathecal calcium-channel blockers, resulting in potent analgesia in this study. Although the reduction in calcium conductance caused by μ and δ receptor activation is coupled to voltage- or calcium-dependent potassium channels, κ receptors are directly coupled to calcium channels, resulting in a decrease of calcium current.[30,31] Therefore, κ receptor agonists may show a different interaction with calcium blockers than μ and δ agonists. Further work is needed to investigate whether calcium-channel blockers are capable of potentiating the analgesia of κ opioid receptor agonists at the level of the spinal cord.

In regard to motor function, calcium-channel blockers alone, at doses employed in the current study, did not show any antinociception; motor reflex of the tail was normally maintained. Furthermore, we assessed the motor function of the hind paws in some rats that were intrathecally administered the combination of morphine and calcium-channel blockers, using Langerman *et al.*'s scale[32] modified for rats. Rats that were administered the combined drugs (2 μg morphine + 50 μg verapamil, 2 μg morphine + 100 μg diltiazem, 2 μg morphine + 20 μg nicardipine) did not show any motor dysfunction. We are, therefore, convinced that intrathecally administered calcium-channel blockers, alone or with morphine, at the doses used have no influence on motor function. However, it is possible that the interation of opioids and calcium-channel blockers produce the potentiation of not only antinociception, but also opioid side effects, including respiratory depression. Further study is needed to investigate the possible potentiation of opioid effects other than antinociception.

Although calcium-channel blockers are known to have vasodilatory and cardiodepressive activities, resulting in hypotension and bradycardia,[33,34] neither intrathecally administered verapamil (200 μg) nor 2 μg morphine combined with 50 μg verapamil produced changes in arterial blood pressure and HR in the current study. It is unlikely, therefore, that systemic hemodynamic changes are the mechanism by which calcium-channel blockers potentiated morphine analgesia in the study. Seyler *et al.*[25] demonstrated that brain levels of morphine in animals treated with morphine only, or morphine plus verapamil, were comparable; and, thus, that verapamil neither influenced the permeability of the blood–brain barrier nor changed morphine distribution to brain. However, there are several alternative explanations for the observed effects. An additional possible mechanism that could explain the observed effects would be a pharmacokinetic interation between the drug solutions, *e.g.*, changes in *p*H of the CSF, changes in spinal cord blood flow, or changes in tail blood flow or temperature. Although we did not examine those possibilities, we need to bear them in mind as we consider drug interations around the spinal cord. Although these and other possibilities exist, the body of evidence supports continued examination of possible antinociceptive interactions between blockers of L-type calcium channels and opioids.

In the current study, we employed three different calcium-channel blockers with different structures; dihydropyridine (nicardipine), diphenylalkylamine (verapamil), and benzothiazepine (diltiazem). The ID_{50} values on the maximum amplitude of the calcium ion inward current in ileum smooth muscle of rabbits were 24 nM, 1.4 μM, and 1.3 μM in nicardipine, diltiazem, and verapamil, respectively.[35] Thus, in spite of the fact that the coadministration doses of calcium-channel blockers administered in the current study may not be equipotent on neuronal calcium ion channels, the synergistic interaction degree (ED_{50} ratio) was comparable between the three blockers. Therefore, it should be stressed that, although the current study provides evidence that the calcium-channel blockers mediate their synergistic actions through neuronal calcium-channel blockade, we do not rule out the possibility that these agents may be acting through more than one mechanism. These agents have been shown the inhibition of adenosine uptake[36] and the inhibition of the calcium dependent regulatory protein calmodulin activity.[37]

Intrathecally or epidurally administered opioids for pain management may be enhanced by the use of concomitant opioids and calcium-channel blockers. The enhancing effects of intrathecal calcium-channel blockers on intrathecal morphine analgesia may be extremely important to minimize doses of opioids and, thus, avoid potential side effects. However, the investigation of the possibility of neurotoxicity to the spinal cord caused by calcium-channel blockers must be conducted before those drugs are administered perispinally to humans.

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