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Antinociceptive action of amlodipine blocking N-type Ca^{2+} channels at the primary afferent neurons in mice

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Abstract

We investigated the antinociceptive action of amlodipine, a dihydropyridine derivative, which acts on both L- and N-type voltage-dependent Ca^{2+} channels (VDCCs), in mice. Intrathecal injection of amlodipine (300 nmol/kg) significantly shortened the licking time in the late phase of a formalin test, while no effect was found with another dihydropyridine derivative, nicardipine (300 nmol/kg). Cilnidipine and ω-conotoxin GVIA also showed marked analgesic effects under the same experimental conditions. Transcripts of α_{1A}, α_{1B}, α_{1E}, α_{1F}, α_{1H}, β3, and β4 subunits were detected by polymerase-chain reaction (PCR) in the dorsal root ganglion, suggesting the existence of a variety of voltage-dependent Ca^{2+} channels. Electrophysiological experiments showed that amlodipine and cilnidipine inhibit N-type currents in the dorsal root ganglion cells. These results suggest that amlodipine, cilnidipine, and ω-conotoxin GVIA exert their antinociceptive actions by blocking N-type Ca^{2+} channels in the primary nociceptive afferent fibers. Blocking of the Ca^{2+} channels results in attenuation of synaptic transmission of nociceptive neurons. Furthermore, it is suggested that some N-type Ca^{2+} channel blockers might have therapeutic potential as analgesics when applied directly into the subarachnoidal space.

Keywords: Ca^{2+} channel; Nociception; Amlodipine

1. Introduction

The dihydropyridines are known to block L-type voltage-dependent Ca^{2+} channels (VDCCs) and are one of the most widely used agents for cardiovascular diseases (Fleckenstein, 1983). Many dihydropyridines cause an apparent increase in heart rate when administered systemically (Lopez et al., 1990). Amlodipine is a dihydropyridine but different from other dihydropyridines in that it causes only a small increase in heart rate. The difference in the effects on the heart rate can be ascribed to the block of N-type Ca^{2+} channels at the sympathetic terminals by amlodipine (Furukawa et al., 1997). It is well known that N-type Ca^{2+} channels are localized at the presynaptic terminals of the neurons, playing important roles in neurotransmitter release (Elliot et al., 1995; Gohil et al., 1994; Hirning et al., 1988; Westenbroek et al., 1992).

The aim of this study was to clarify the antinociceptive action of amlodipine in mice. For this purpose, we delivered amlodipine intrathecally and examined the behavioral responses to nociceptive stimulation with formalin. We also examined the analgesic action of cilnidipine, which acts on both L- and N-type Ca^{2+} channels, nicardipine, which is a specific L-type channel blocker, and ω-conotoxin GVIA, a specific blocker of N-type channels (Hil-

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lyard et al., 1992; McCleskey et al., 1987). In order to investigate the subtypes of Ca^{2+} channels involved in nociceptive transduction in dorsal root ganglion, we analyzed the expression profile of the subunits of Ca^{2+} channels in the dorsal root ganglion by reverse transcription-polymerase-chain reaction (PCR). Furthermore, patch-clamp experiments were done with isolated neurons from the dorsal root ganglion to evaluate the inhibitory effects of these dihydropyridines on N-type Ca^{2+} channels.

2. Materials and methods

2.1. Drugs

ω-Conotoxin GVIA, calciseptine, and ω-agatoxin IVA were purchased from the Peptide Institute (Minoh, Japan). Amlodipine and cilnidipine were kindly supplied by Fuji-Rebio (Tokyo, Japan). Collagenase and 1,2-bisethane-,*N*,*N*,*N*′,*N*′,-tetra acetic acid (BAPTA) were purchased from Wako (Osaka, Japan) and Dojin (Kumamoto, Japan), respectively. All other chemicals were purchased from Sigma (St. Louis, USA) and were of reagent grade.

Amlodipine, ω-conotoxin GVIA, calciseptine, and ω-agatoxin IVA were dissolved in distilled water. Cilnidipine and nicardipine were dissolved in dimethyl sulfoxide (DMSO) at 10 mM as stock solutions.

2.2. Animals

The subjects were experimentally naive, 4- to 5-week-old mice held at 22 ± 0.5°C with a 12-h light–dark cycle. All animals were used only once. All experiments were conducted during the light phase of the cycle. The behavioral experiments were performed with the approval of the ethics committee for animal experiments of Tohoku Pharmaceutical University.

2.3. Formalin test

Intrathecal injections were made in unanesthetized mice at the intervertebral L5 and L6 space by the technique of Hylden and Wilcox, 10 min prior to the formalin test, as previously reported (Sakurada et al., 1995). A volume of 5 μl was injected intrathecally with a 28-gauge needle connected to a 50-μl Hamilton micro-syringe while the animal was lightly restrained to maintain the position of the needle. Compounds were dissolved in sterile artificial cerebrospinal fluid, which consisted of (in mM) 126.6 NaCl, 2.5 KCl, 2.0 $MgCl_2$, and 1.3 $CaCl_2$. The artificial cerebrospinal fluid was injected in the control experiments.

The formalin test was done by subcutaneously injecting 20 μl of a 2.0% formaldehyde solution under the dorsal surface of the right hind paw. After the injection, behavior was observed and the nociceptive response was recorded during the early phase (0–10 min after injection) and late phase (10–30 min after injection). The time spent licking the injected paw was calculated as an indicator of nociception.

2.4. RNA isolation and reverse transcription (RT)-PCR analysis

Total RNA was isolated from the mouse dorsal root ganglion by RNeasy extraction kit (Qiagen Valencia, USA). The reverse transcription reaction was performed in a volume of 25 μl containing 10 pmol oligo-dT primer, 1 μg RNA, 1× first strand cDNA buffer (Life Technologies, Rockville, USA), 10 mM dithiothreitol, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, 0.4 mM dTTP, 40 U RNAsin, and 200 U Superscript II (Life Technologies), at 42°C for 45 min. A 2-μl portion of the mixture of the reverse transcription reaction was used for the PCR assays. α_{1A}, α_{1B}, α_{1F}, α_{1E} and α_{1H} subunit specific sequences were amplified by PCR with the primers: A1A1 (5′-CTC CCG AGA ACA GCC TTA TC-3′) and A1A2 (5′-GTC TGC CTC CTC TTC CTC TTT CTT C-3′), which correspond to the sequences of the murine α_{1A} subunit, T_{403} PENSLIVT$_{411}$ and E_{463}KKEEEEAD$_{471}$; A1B1 (5′-GGG GAT AAG GAA ACC CGA AAT CAC CA-3′) and A1B2 (5′-CTT GGC CTT CCA GGT TCA TGT TAC CA-3′), which correspond to the sequences of the murine α_{1B} subunit, G_{319}DKETRNHQ$_{327}$ and G_{403}NMNLEGQA$_{411}$; A1E1 (5′-AGA CAC CAC ATG TCG ATG TGG-3′) and A1E2 (5′-GTT TCC ATG ACA GGA TCT AGG-3′), which correspond to the sequences of the murine α_{1E} subunit $_{754}$RHHMSMW$_{761}$ and $_{885}$PRSCHGN$_{892}$; MF1 (5′-ACT GCC AAA GAT AAG GGC AGA-3′) and MF2 (5′-ATG TAT GAG TGT GTG GCA GGC-3′), which correspond to the sequences of the murine α_{1F} subunit, T_{768}AKDKGR$_{774}$ and A_{868}CHTLIH$_{874}$; MH1 (5′-ATG TAC TCA CTG GCT GTG ACC-3′) and MH2 (5′-TGA GTT TGG TCT GCT GTC CTC-3′), which correspond to the sequences of the murine α_{1H} subunit, M_{939}YSLAVT$_{945}$ and E_{1065}DSRPNS$_{1071}$. β3 and β4 subunit specific sequences were amplified by PCR with specific primers, MB3S (5′-C TC AAA CAG GAA CAG AAG GCC-3′) and MB3A (5′-CAT AGC CTT TCA GAG AGG GTC-3′), which correspond to the sequences of the murine β3 subunit L_{129}KQEQKAR$_{135}$ and P_{186}SLKGYE$_{192}$; MB4S (5′-CTG AGC CTT TCA GAG AGG GTC-3′) and MB4A (5′-CAT TGA CGG CAC GAC GTC ATA-3′), which correspond to the sequences of the murine β4 subunit L_{154}RLENIR$_{160}$ and Y_{213}DVVPSM$_{219}$.

2.5. Electrophysiological experiments

Dorsal root ganglion cells were isolated from 1- to 5-day-old Wistar rats. After chemical digestion in Ringer

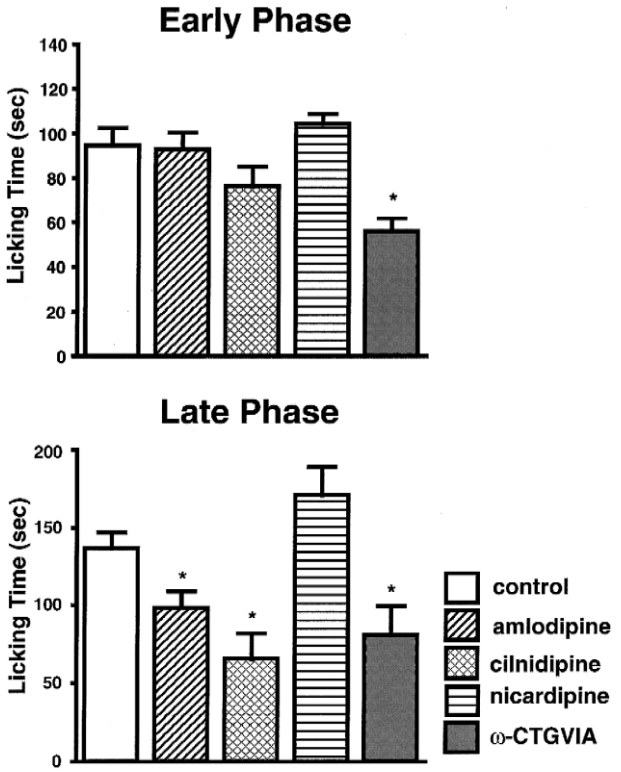

Fig. 1. The effect of intrathecal pretreatment with amlodipine (300 nmol/kg), cilnidipine (300 nmol/kg), nicardipine (300 nmol/kg), and ω-conotoxin GVIA (0.46 nmol/kg) on the behavior induced by subcutaneous formalin injection. Nociceptive behavior in both the early (0–10 min; upper panel) and late (10–30 min; lower panel) phases is shown as the time spent licking the injected paw. The data are expressed as the means ± S.E.M. Student's *t*-test was used to isolate differences from control. Differences were considered significant when $P < 0.01$ (asterisk).

solution containing 0.1% collagenase and 0.05% trypsin at 37°C for 30 min, the dorsal root ganglions were rinsed twice with 2-ml enzyme-free Ringer solution. Single neurons were obtained by trituration through a fire-polished Pasteur pipette. The neurons obtained were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 ng/ml 7S nerve growth factor, 50 μg/ml streptomycin sulfate, and 50 U/ml penicillin G. They were then plated onto glass coverslips coated with poly-L-lysine. Cultures were maintained at 37°C in humidified air containing 5% CO_2. Non-neuronal cell proliferation was reduced by the addition of 5 mM cytosine β-D-arabinofuranoside for the first 24 h.

Currents were recorded in a whole-cell configuration from single cell bodies of dorsal root ganglion neurons using a voltage-clamp amplifier (CEZ-2200, Nihon Khoden, Tokyo, Japan), as previously described (Fujii et al., 1997). Currents were filtered at 3 kHz and stored in a computer (7100/80 AV, Apple Computer, Cupertino, USA) through an AD/DA interface (ITC-16, Instrutech Greatneck, New York, USA; sampling rate 10 kHz) using Axodata software (v1.2, Axon Instrument, Foster, USA). The pipette solution consisted of (in mM) 135 CsCl, 5 $MgCl_2$, 5 BAPTA, 10 HEPES, 5 ATP, and 12 glucose (pH 7.0 with CsOH). The bath solution consisted of (in mM) 100 Tris, 5 CsCl, 5 $BaCl_2$, 1 $MgCl_2$, 25 Tetraethylammonium-Cl, 5 HEPES, and 20 glucose (pH 7.4 with Tris–HCl). Currents were evoked by step depolarization to 0 mV from a holding potential of −60 mV (30 ms duration, 0.18 Hz). All experiments were performed at room temperature (23–26°C). Capacitative and leak currents were subtracted using the P/4 method. The depolarization-induced Ba^{2+} current was measured at peak. Drug action was assessed by calculating the difference between the peak current amplitudes before and 3–4 min after the application of each drug. Amlodipine, cilnidipine, and nicardipine were diluted to the final concentrations with the bath solution and applied by superfusion. ω-Conotoxin GVIA, calciseptine and ω-agatoxin IVA were delivered through a pressure-ejection pipette at the concentrations indicated.

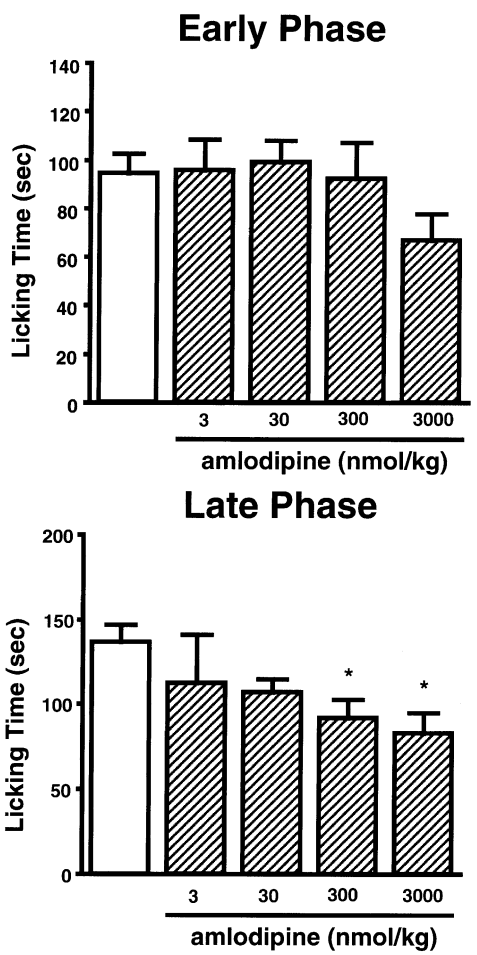

Fig. 2. The effect of amlodipine on the duration of licking after subcutaneous formalin injection. The open and hatched columns represent control and amlodipine, respectively. The data are expressed as the means ± S.E.M. Dunnett's multiple comparison test was used to evaluate the difference between control and amlodipine. Differences were considered significant when $P < 0.01$ (asterisk).

2.6. Data analysis

Data are expressed as means ± S.E.M. The significance of differences between two groups was determined with Student's *t*-test. Dunnett's multiple comparison was used for the multiple intergroup test.

3. Results

3.1. Antinociceptive effects of amlodipine, cilnidipine, and ω-conotoxin GVIA

Intrathecal injection of amlodipine (300 nmol/kg) 10 min prior to subcutaneous formalin significantly reduced the licking time in the late phase (Fig. 1). The effect of amlodipine on the licking time in the late phase was dose-dependent, whereas amlodipine had no significant effect in the early phase even at the highest dose (Fig. 2). Cilnidipine (300 nmol/kg) also produced a significant decrease in licking time in the late phase of the formalin test. Intrathecal injection of ω-conotoxin GVIA (0.46 nmol/kg), which is a specific blocker of N-type channels and known to have an analgesic action (Hillyard et al., 1992; Malmberg and Yaksh, 1994, 1995; McCleskey et al., 1987; Olivera et al., 1984), significantly decreased licking time in both the early and the late phases. On the other hand, nicardipine showed no apparent inhibitory effect in either phase at the same dose as amlodipine and cilnidipine.

3.2. The subunits of the voltage-dependent Ca2+ channels expressed in the dorsal root ganglion

To investigate the expression of the subunits of the voltage-dependent Ca^{2+} channel, we analyzed RNA transcripts of the dorsal root ganglion by reverse transcription-PCR. Fragments of 204, 276, 417, 321, 396, 187 and 195 bp were generated, which correspond to the α_{1A}, α_{1B}, α_{1E}, α_{1F}, α_{1H}, β3 and β4 subunit specific fragments, respectively (Fig. 3). The results of the reverse transcription-PCR analysis suggested the presence of various types of Ca^{2+} channels, such as N-, P/Q- and L-type Ca^{2+} channels in the dorsal root ganglion.

3.3. Effects of the dihydropyridines on N-type Ca2+ currents

The effect of amlodipine on N-type Ca^{2+} channels was examined in the dorsal root ganglion neurons in the presence of 3 μM calciseptine and 2 μM ω-agatoxin IVA, which specifically block L- and P/Q-type channels, respectively. Fig. 4A shows representative current traces revealing the inhibitory effect of amlodipine on the N-type current. To compare the effects of amlodipine, cilnidipine, and nicardipine on the N-type channels, the reduction of the current amplitude observed with the dihydropyridines

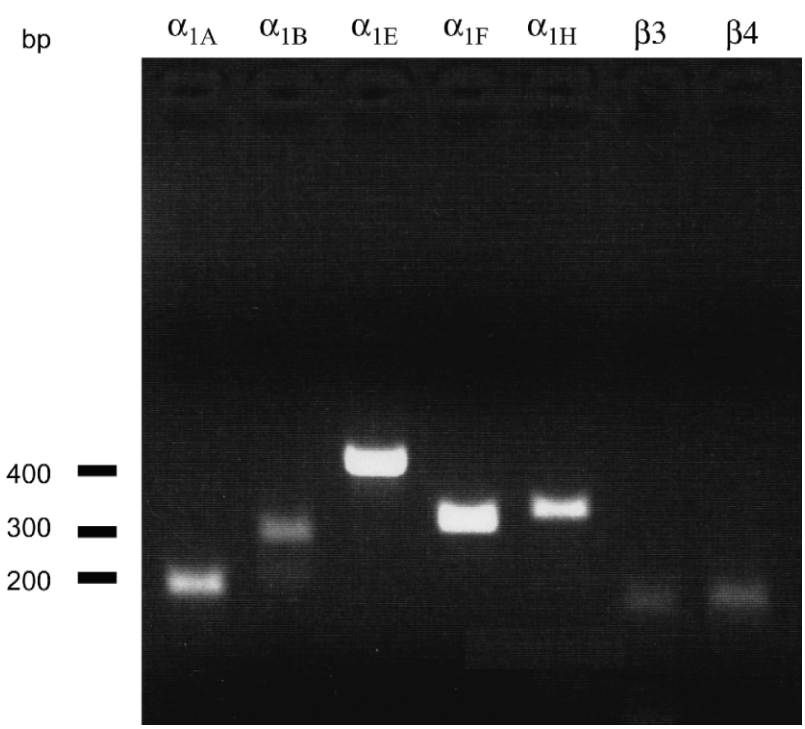

Fig. 3. Expression of α_{1A}, α_{1B}, α_{1E}, α_{1F}, α_{1H}, β3 and β4 subunits of the voltage-dependent Ca^{2+} channels in murine dorsal root ganglion. The presence of 204, 276, 417, 321, 396, 187, and 195 bp fragments, which correspond to the α_{1A}, α_{1B}, α_{1E}, α_{1F}, α_{1H}, β3 and β4 subunits, respectively, was revealed in the dorsal root ganglion by RT-PCR analysis.

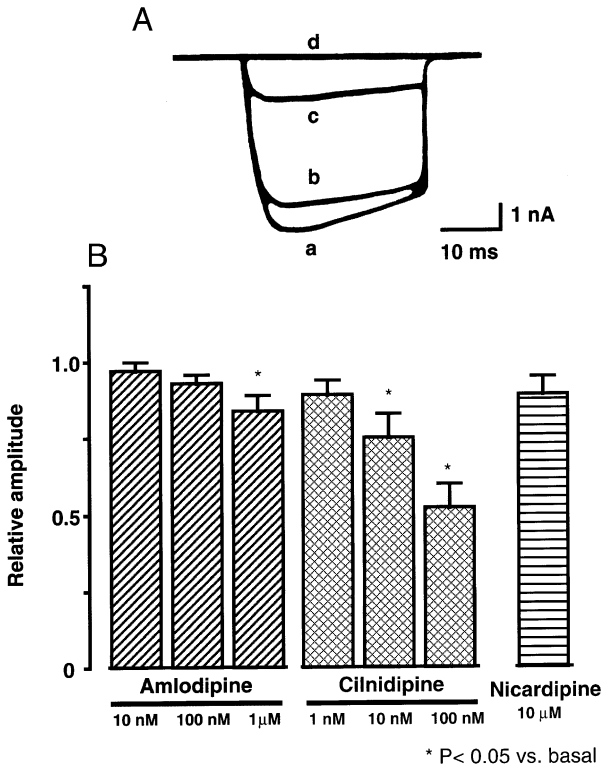

Fig. 4. Effects of amlodipine (10–1000 nM), cilnidipine (1–100 nM) and nicardipine (10 μM) on the N-type current in rat dorsal root ganglion neurons in the presence of 3 μM calciseptine and 2 μM ω-agatoxin IVA. Amlodipine was added to the superfusate. (A) Current traces are as follows: a, control; b, 1 μM amlodipine; c, 3 μM ω-conotoxin GVIA; d, 100 μM Cd^{2+}. The currents were evoked by depolarizing pulses to 0 mV from a holding potential of −60 mV (30 ms duration, 0.18 Hz). The current was almost abolished with 3 μM ω-conotoxin GVIA, indicating that the currents observed were N-type ones. (B) The current amplitudes observed in the presence of amlodipine, nicardipine, and cilnidipine. To evaluate the effects of each drug, the reduction of the current amplitude induced by 3 μM ω-conotoxin GVIA was normalized as 1.0, and the percent reduction relating to the ω-conotoxin GVIA (3 μM)-sensitive current was examined. Each column and vertical bar represents the mean and S.E.M from three to seven different neurons, respectively.

was normalized to that induced by 3 μM ω-conotoxin GVIA. The concentrations of ω-conotoxin GVIA (≥ 3 μM) used in our study have been reported to selectively block N-type channels (Aosaki and Kasai, 1989; Bleakman et al., 1995; Wheeler et al., 1994). The highest dose of amlodipine (1 μM) reduced the peak amplitude by about 20%. The same dose of cilnidipine also reduced the amplitude by about 50%, whereas only a very high concentration of nicardipine (10 μM) inhibited the ω-conotoxin GVIA-sensitive current by about 10% (Fig. 4B). Compared with amlodipine, cilnidipine was more potent to block N-type Ca^{2+} channels, with an approximately 50% reduction of the N-type current at a concentration of 100 nM. This was similar to the IC_{50} of cilnidipine for L-type channels (IC_{50} = 100 nM)(Fujii et al., 1997).

4. Discussion

The Ca^{2+} channel blockers have been widely used for cardiovascular diseases but are scarcely used for neurological disorders (Fleckenstein, 1983; Triggle, 1990, 1999). In this study, we have demonstrated that acute intrathecal injection of amlodipine results in a significant decrease in the licking time during the late phase of the formalin test. Electrophysiological examination revealed the blocking action of amlodipine on the N-type Ca^{2+} channels in the dorsal root ganglion neurons. These findings suggest that amlodipine has an antinociceptive action, which can be ascribed to its blocking of N-type Ca^{2+} channels.

Amlodipine is one of the most commonly used agents for hypertension in Japan (Saruta, 1998). Amlodipine blocks the N-type Ca^{2+} channels as well as the L-type channels (Furukawa et al., 1997). Its IC_{50} values for the L- and N-type channels in a *Xenopus* oocyte system are 2.4 and 5.8 μM, respectively (Furukawa et al., 1997). Since ω-conotoxin GVIA has an analgesic action and amlodipine shows similar IC_{50} values for L- and N-type channels, we investigated the analgesic effect of amlodipine and compared it with the effect of other L/N-type Ca^{2+} channel blockers, cilnidipine and ω-conotoxin GVIA.

It has been reported that there are a number of different high-voltage-activated Ca^{2+} channels, L-, N-, P/Q-, R- and T-types, in the dorsal root ganglion (Fujii et al., 1997; Diochot et al., 1995; Scroggs and Fox, 1992). In the reverse transcription-PCR analysis, we confirmed the expression of the five pore-forming α_1 subunits, α_{1A}, α_{1B}, α_{1E}, α_{1F}, and α_{1H}. Not all of the corresponding channels are completely well characterized, but as these α_1 subunits are thought to form P/Q-, N-, R-, L- and T-type Ca^{2+} channels (Hofmann et al., 1994; Lee et al., 1999), the expression of the subunits corresponds to the existence of N-, P/Q-, R-, L- and T-type channels in the dorsal root ganglion. Of these channels, N- and P/Q-type channels are localized at the presynaptic terminal of the neuron and mediate Ca^{2+} influx, which is necessary for the release of neurotransmitters. It has been shown that blockade of N-type channels of presynaptic terminals by ω-conotoxin GVIA inhibits glutaminergic transmission between the neurons of the dorsal root ganglion and the spinal cord (Gruner and Silva, 1994). Intrathecal administration of ω-conotoxin GVIA and ω-agatoxin IVA inhibited the excitability of the dorsal horn neurons, but L-type Ca^{2+} channel blockers did not in a formalin model (Diaz and Dickenson, 1997). The blocking action of N-type channel blockers on the release of transmitter in the dorsal root ganglion results in decreased nociceptive transmission. Considering the expression of the N-type Ca^{2+} channels in the dorsal root ganglion and the blocking action of amlodipine and cilnidipine on the N-type current, it is likely that these dihydropyridines exert their analgesic action by blocking Ca^{2+} influx through the N-type channels. From the results of the formalin test, the rank order of potency of

analgesic action was as follows: ω-conotoxin GVIA ≫ cilnidipine > amlodipine, while nicardipine had no effect. The rank order of potency of the agents for blocking the N-type current was the same as that of the analgesic action. These results further support the notion that the analgesic action of the dihydropyridines is probably due to their blocking actions on N-type currents of the neurons in the central nervous system, including the dorsal root ganglion ones, resulting in decreased synaptic transmission of the nociceptive pathway.

Although amlodipine blocks the N-type channels, there are some differences in the effects of amlodipine and ω-conotoxin GVIA. While amlodipine showed an effect only in the late phase of the formalin test, ω-conotoxin GVIA showed an inhibitory effect in both the early and late phases. Formalin produces nociceptive stimuli and provides a model of chemical stimulation. In contrast to other kinds of stimuli such as thermal, mechanical, and visceral, the response to the stimulation by formalin comprises two phases. The early phase corresponds to the response to direct stimulation of the nerve endings, and the late phase results from the subsequent inflammation (Dubuisson and Dennis, 1977). Because amlodipine and cilnidipine showed significant effects on the late phase, we think they act only on the inflammatory nociception.

Ca^{2+} channels are composed of heteromeric multi-subunits. β subunits are the most important auxiliary subunits because they modify activation, inactivation, and channel population (Hofmann et al., 1994). Analysis of the expression of β subunits revealed that only the β3 and β4 subunits were detected in the neurons of dorsal root ganglion. The results have suggested that the β3 and β4 subunits are expressed in the dorsal root ganglion and form Ca^{2+} channels in association with the α_{1A}, α_{1B}, α_{1E}, or α_{1F} subunits in the dorsal root ganglion.

In summary, we have shown that intrathecal injection of amlodipine results in a slight but significant reduction of the response in the late phase of the formalin test in mice. Amlodipine also showed a slight but significant inhibitory action on N-type currents in rat dorsal root ganglion neurons. We also investigated the effects of other dihydropyridines on the response to nociceptive stimulation and on the N-type Ca^{2+} current. There was a correlation between the analgesic effects and the inhibitory action on N-type currents in the dorsal root ganglion. It is suggested that some of the dihydropyridines show an analgesic action, probably through blockade of N-type Ca^{2+} channels of the neurons in the nociceptive pathway. Furthermore, N-type Ca^{2+} channel blockers may have potential as analgesics when applied directly to the central nervous system.

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