Selectivities of Dihydropyridine Derivatives in Blocking Ca\(^{2+}\) Channel Subtypes Expressed in Xenopus Oocytes\(^1\)

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ABSTRACT

Some dihydropyridines (DHPs), such as amlodipine and cilnidipine, have been shown to block not only L-type but also N-type Ca\(^{2+}\) channels; therefore, DHPs are no longer considered as L-type-specific Ca\(^{2+}\) channel blockers. However, selectivity of DHPs for Ca\(^{2+}\) channel subtypes including N-, P/Q-, and R-types are poorly understood. To address this issue at the molecular level, blocking effects of 10 DHPs (nifedipine, nilvadipine, barnidipine, nimodipine, nitrendipine, amlodipine, nicardipine, and cilnidipine) on four subtypes of Ca\(^{2+}\) channels (L-, N-, P/Q-, and R-types) were investigated in the Xenopus oocyte expression system with the use of the two-microelectrode voltage-clamp technique. L-type Ca\(^{2+}\) channels expressed as \(\alpha_{1C}\beta_{1A}\) combination were profoundly blocked by all DHPs examined, whereas blocking actions of these DHPs on R-type \(\alpha_{1E}\beta_{1A}\) channels were equally weak. In contrast, 5 of the 10 DHPs (amlodipine, benidipine, cilnidipine, nicardipine, and barnidipine) significantly blocked N-type \(\alpha_{1B}\beta_{1A}\) and P/Q-type \(\alpha_{1A}\beta_{1A}\) Ca\(^{2+}\) channels. These selectivities of DHPs in blocking Ca\(^{2+}\) channel subtypes would provide useful pharmacological and clinical information on the mode of action of the drugs including side effects and adverse effects.

High voltage-activated Ca\(^{2+}\) channels in excitable cells such as myocytes, smooth muscle cells, and neurons play important roles, including contraction of myocytes, electrical excitation in neurons, and modulation of hormone and neurotransmitter release (Tsien et al., 1991). High voltage-activated Ca\(^{2+}\) channels are pharmacologically classified into at least five different subclasses (L-, N-, P-, Q-, and R-type), the characteristics of which are determined by the pore-forming \(\alpha_1\) subunit. The subunits \(\alpha_{1C}\), \(\alpha_{1D}\), and \(\alpha_{1E}\) form L-type Ca\(^{2+}\) channels and bind dihydropyridines (DHPs), phenylalkylamines, and benzothiazepines with high affinity, whereas the subunits \(\alpha_{1B}\), \(\alpha_{1A}\), and \(\alpha_{1E}\) form N-, P/Q-, and R-type Ca\(^{2+}\) channels, respectively, which show low affinities for these drugs (Hockerman et al., 1997; Hering et al., 1998; Striessnig et al., 1998). Because nifedipine, the prototype of the DHPs, exclusively blocked muscular L-type Ca\(^{2+}\) channels (Fleckenstein, 1983), DHPs had been considered as selective blockers for L-type channels.

Recent studies have shown, however, that two DHPs, amlodipine (Furukawa et al., 1997) and cilnidipine (Fuji et al., 1997; Uneyama et al., 1997), blocked N-type Ca\(^{2+}\) channels as well. These findings indicate that DHPs are no longer considered L-type specific blockers, and suggest that some DHPs may block other subtypes of Ca\(^{2+}\) channels, such as P/Q-, and R-types. DHPs are widely used clinically in the treatment of hypertension, angina pectoris, and cerebrovascular diseases. However, pharmacological profiles of the effects of DHP on each Ca\(^{2+}\) channel subtype are not understood well enough for them to be used with confidence with these drugs.

Non-L-type Ca\(^{2+}\) channels are diversely distributed in peripheral and central nervous cells (Tsien et al., 1991). However, native neuronal cells and cell lines possess multiple subtypes of Ca\(^{2+}\) channels in a single cell, which hampers quantitative comparison of effects of a given drug on a single subtype of Ca\(^{2+}\) channel. To address these issues at the molecular level, a single subclass of the Ca\(^{2+}\) channel \(\alpha_1\) subunit \(\alpha_{1A}\), \(\alpha_{1B}\), \(\alpha_{1C}\), or \(\alpha_{1E}\) was coexpressed with the same auxiliary \(\alpha_2\) and \(\beta\) subunits in Xenopus oocytes, and 10 DHP derivatives used clinically were examined for their channel-blocking effects.

Materials and Methods

Methods for in vitro transcription of cRNAs specific to the Ca\(^{2+}\) channel \(\alpha_1\), \(\alpha_2\), and \(\beta_{1A}\) subunits and procedures for functional ex-

ABBREVIATIONS: DHP, dihydropyridine; DMSO, dimethyl sulfoxide; I-V, current-voltage; IC\(_{50}\), concentration at half-blockade.
pression of Ca^{2+} channels in Xenopus oocytes were described previously (Furukawa et al., 1998). After removal of the follicular cell layer, Xenopus oocytes were injected with 0.3 μg/μl α1a (Mikami et al., 1989), α1b (Fujita et al., 1993), α1a (Mori et al., 1991), or α1E (Niidome et al., 1992) cRNA in combination with 0.2 μg/μl α2 (Mikami et al., 1989) cRNA and 0.1 μg/μl β1a (Mori et al., 1991) cRNA. In some experiments, the cRNA for the β3b or β3 subunit (Hullin et al., 1992) was used instead of that for β1a subunit.

The oocytes were cultured for 2 to 4 days and then subjected to electrophysiological measurement. The oocytes were placed in a small chamber perfused with extracellular solution (10 mM Ba^{2+}, 90 mM Na^{+}, 2 mM K^{+}, 5 mM HEPES, and 0.3 mM niflumic acid, pH 7.5, with methanesulfonic acid), and Ba^{2+} currents through expressed channels were measured by the two-microelectrode voltage-clamp method with a GeneClamp 500 amplifier (Axon Instruments, Foster City, CA). The experimental chamber was 0.5 ml in volume, and it was perfused continuously (2.0–3.0 ml/min) with the extracellular solution. Commercial software (pClamp version 6.0; Axon Instruments) was used for generating voltage pulses, acquiring data, and analyzing the currents. Typically, oocytes were clamped at a holding potential of −100, −80, or −60 mV and depolarized to +10 mV for 200 ms every 15 s. Microelectrodes were filled with 3 M KCl, and those showing a resistance of 0.5 to 1.2 ΩM were used.

The drug effects were evaluated after a 5-min perfusion of bath solution containing a DHP derivative. In experiments to obtain concentration-response relationships, the concentrations of the DHPs were changed successively. Each experiment was finished within 20 min to avoid possible run down of Ba^{2+} currents. Because no detectable current change was observed during exposure to the vehicle for DHP [0.2% dimethyl sulfoxide (DMSO)] as reported previously (Furukawa et al., 1998), injection of the DMSO into the bath solution to make the final concentration. The following DHPs DHP [0.2% dimethyl sulfoxide (DMSO)] as reported previously (Furukawa et al., 1998). After removal of the follicular cell

### Results

As reported previously (Furukawa et al., 1998), injection of cRNAs specific for Ca^{2+} channel α1 subunit (α1B, α1A, or α1C) in combination with cRNAs for the Ca^{2+} channel α2 and β1a subunits resulted in functional expressions of Ca^{2+} channels that possessed the native characteristics of ω-conotoxin GVIA-sensitive N-type, α-agatoxin IVA-sensitive P/Q-type, and nifedipine-sensitive L-type channels, respectively. In addition, N-type (α1Bα2β1a) and P/Q-type (α1Aα2β1a) channels were not blocked by 10 μM nifedipine (Furukawa et al., 1998). In oocytes injected with cRNAs for Ca^{2+} channel α1B, α2, and β1a subunits, inward currents through α1Bα2β1a channels were not blocked by any of these toxins or nifedipine (n = 5), which is a characteristic feature of the R-type Ca^{2+} channel (Birnbaumer et al., 1994).

To conduct an effective screening of the selectivities of the 10 DHP derivatives (nifedipine, nilvadipine, barnidipine, nifedipine, nitrendipine, amlodipine, nicardipine, benidipine, felodipine, and cilnidipine) in blocking each subtype of Ca^{2+} channel (Table 1; Fig. 1), we first examined the effects of 10 μM DHPs on the channel subtypes at a holding potential of −80 mV (Fig. 2). All of the DHPs examined inhibited L-type (α1Cα2β1a) Ca^{2+} channels by 30 to 65%, whereas none of them blocked R-type (α1Bα2β1a) channels more than 10%. In contrast to R-type channels, N-type (α1Aα2β1a) and P/Q-type (α1Aα2β1a) channels were appreciably blocked by 5 DHPs among the 10 tested (barnidipine, amlodipine, nicardipine, benidipine, and cilnidipine). The blocking action of these DHPs on N- and P/Q-type channels was not related to the blocking potency for L-type channels. Moreover, each DHP shared a blocking action on both N- and P/Q-type channels. The remaining DHPs (nifedipine, nilvadipine, nimodipine, nitrendipine, and felodipine) scarcely blocked the two channel subtypes. In the case of amlodipine, N-type channels were more profoundly inhibited than were L- and P/Q-type channels. Thus, barnidipine, amlodipine, nicardipine, benidipine, and cilnidipine were not categorized as L-type-selective DHPs, and their effects on N- and P/Q-type channels were investigated in more detail.

The Ca^{2+} channel β subunit is known to modulate channel kinetics and DHP sensitivity, and we used the skeletal muscle form of the β subunit. To get insight into the modulation of DHP effect by β subunit subclass, we compared the effect of amlodipine on α1Bα2β1A, α1Aα2β2A, and α1Eα2β3 channels. The block of Ba^{2+} current at a holding potential of −80 mV by 10 μM amlodipine were 78.4 ± 7.2 (n = 12) for the α1Bα2β1A channel, 69.6 ± 10.2 for the α1Aα2β2A channel (n = 7), and 71.2 ± 13.2 for the α1Eα2β3 channel (n = 8), respectively. The blocking actions of amlodipine on N-type Ca^{2+} channels with different β subunit subclasses were not substantially different.

The blockade of L-type Ca^{2+} channels by amlodipine is known to develop slowly (Kass and Arena, 1989). Under the present experimental conditions (at a holding potential of

### TABLE 1

Basic properties of the 10 DHPs

<table>
<thead>
<tr>
<th>DHP</th>
<th>MW</th>
<th>Partition Coefficient</th>
<th>pK_{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nifedipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>346.3</td>
<td>9,000</td>
</tr>
<tr>
<td>Nilvadipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>348.4</td>
<td>14,000</td>
</tr>
<tr>
<td>Barnidipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>489.5</td>
<td>&gt;2,000</td>
</tr>
<tr>
<td>Nimodipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>489.5</td>
<td>2,650</td>
</tr>
<tr>
<td>Nitrendipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>360.4</td>
<td>13,000</td>
</tr>
<tr>
<td>Amlodipine</td>
<td>C_{16}H_{18}ClN_{2}O_{5}</td>
<td>567.1</td>
<td>26.1</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>514.0</td>
<td>16.1</td>
</tr>
<tr>
<td>Benidipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>542.0</td>
<td>6,460</td>
</tr>
<tr>
<td>Felodipine</td>
<td>C_{16}H_{18}ClN_{2}O_{5}</td>
<td>384.3</td>
<td>Not soluble in water</td>
</tr>
<tr>
<td>Cilnidipine</td>
<td>C_{16}H_{18}N_{2}O_{5}</td>
<td>492.5</td>
<td></td>
</tr>
</tbody>
</table>

* From drug interview forms.

* MW, molecular weight.
280 mV), the time constant of blocking by 10 mM amlodipine was 42.8 ± 7.8 s (n = 8) for an N-type channel or 45.8 ± 5.5 s (n = 6) for a P/Q-type channel. At a holding potential of −100 mV, the time constant of blocking for an N-type Ca\(^{2+}\) channel was 1 min (Furukawa et al., 1997). Considering these time constants of blocking, we determined that perfusion of amlodipine for 5 min was enough to reach steady-state blocking. Blockades of N- and P/Q-type channels by other DHPs developed much faster, and a steady-state inhibition was reached within 1 min (n = 5–12).

Figure 3 shows the effects of the aforementioned five DHPs on the current-voltage (I-V) relationships of N-type Ca\(^{2+}\) channels. These DHPs blocked Ba\(^{2+}\) currents through the N-type (\(\alpha_{1B}\alpha_{2}\beta_{1a}\)) channels at each membrane potential without shifting peak I-V relationships. Similar results were obtained for P/Q-type (\(\alpha_{1A}\alpha_{2}\beta_{1a}\)) Ca\(^{2+}\) channels (Fig. 4).

In the next step, we investigated the concentration- and voltage-dependent effects of these five DHPs on L-, N-, and P/Q-type channels. The oocytes expressing L-type Ca\(^{2+}\) channel were clamped at holding potentials of −80 and −60 mV, and those expressing N- or P/Q-type Ca\(^{2+}\) channels were clamped at holding potentials of −100, −80, and −60 mV. We then measured the blockade of Ba\(^{2+}\) currents by DHPs at various concentrations. Figures 5 through 9 summarize the effects of DHP derivatives on these three subtypes of Ca\(^{2+}\) channels. To describe the concentration-response relationships in a quantitative way, we performed a least-squares fit to data as follows:

\[
\text{Block(\%)} = 100/(1 + (\text{IC}_{50}/[D])^{n_H})
\]

where \(\text{IC}_{50}\) is the concentration at half-blockade, [D] is the drug concentration, and \(n_H\) is the Hill coefficient. All five DHPs showed a concentration-dependent blocking of both N- and P/Q-type Ca\(^{2+}\) channels. In the case of N-type channels (Figs. 5–9, left), the blockade by these DHPs was more potentiated by depolarization of the holding potential, and the voltage-dependence of blocking was not prominent for benidipine, although it was still significant (Fig. 6, left). In contrast to N-type channels, P/Q-type channels were not blocked by benidipine or cilnidipine in a voltage-dependent manner (Figs. 6 and 9, right). Moreover, blockades of P/Q-type channels by amlodipine, nicardipine, and barnidipine were less voltage-dependent than were those of N-type channels (Figs. 5, 7, and 8, right). Amlodipine, barnidipine, and nicardipine had simple concentration- and voltage-dependent blocking actions on L-type Ca\(^{2+}\) channels. However, the effect of benidipine and cilnidipine was variable and complex. The effect of benidipine on an L-type Ca\(^{2+}\) channel was variable as shown by the S.E. bars in Fig. 6. Depolarizing the holding potential decreased the block at drug concentrations of 1 to 10 μM, and benidipine did not block the Ba\(^{2+}\) current completely even at a very high concentration (30 μM) and at a holding potential of −60 mV. Moreover, cilnidipine at concentrations of 1 to 10 μM enhanced the Ba\(^{2+}\) current in some experiments. The Ba\(^{2+}\)

\[\text{Fig. 1. Structures of the 10 DHPs tested.}\]
Fig. 3. Effects of five DHPs on I-V relationships of N-type Ca^{2+} channels. Membrane currents were elicited by step depolarizations of 200-ms duration from holding potential of -80 to +50 or +70 mV every 10 mV. Membrane currents in response to the step pulse to +10 mV before (Control) and after 5-min perfusion of barnidipine (A), amlodipine (B), nicardipine (C), benidipine (D), or cilnidipine (E) are presented with current traces (left) and I-V curves of peak currents (right). Note that these DHPs reduced the membrane currents at each test pulse potential.
Fig. 4. Effects of five DHPs on $I-V$ relationships of P/Q-type Ca$^{2+}$ channels. The same protocols as in Fig. 3 were used. Membrane currents before (Control) and after 5-min perfusion of barnidipine (A), amlodipine (B), nicardipine (C), benidipine (D), or cilnidipine (E) are presented with current traces (left) and $I-V$ curves of peak currents (right). Note that these DHPs also reduced the membrane currents at each test pulse potential, as observed in the case of N-type channels (Fig. 2).
Selectivities of DHPs for Ca\(^{2+}\) Channel Subtypes

**Barnidipine Effect**

- **L-type**
  - Concentration-response curves for barnidipine at holding potentials of \(-100 (○), -80 (■), \) and \(-60 \text{ mV (▲)}\). The IC\(_{50}\) and n\(_{H}\) for an L-type Ca\(^{2+}\) channel are \(3.1 \pm 0.8 \mu\text{M}\) and \(0.5 \pm 0.1 \text{ at } -80 \text{ mV (n = 7)}, \) and \(1.2 \pm 0.3 \mu\text{M}\) and \(0.7 \pm 0.1 \text{ at } -60 \text{ mV (n = 6)}, \) respectively. Those for an N-type Ca\(^{2+}\) channel are \(1370 \pm 499 \mu\text{M}\) and \(0.4 \pm 0.1 \text{ at } -100 \text{ mV (n = 6)}, \) \(74.9 \pm 29.2 \mu\text{M}\) and \(0.4 \pm 0.2 \text{ at } -80 \text{ mV (n = 6)}, \) and \(7.1 \pm 2.6 \mu\text{M}\) and \(0.6 \pm 0.1 \text{ at } -60 \text{ mV (n = 6)}, \) respectively. Those for a P/Q-type Ca\(^{2+}\) channel are \(213 \pm 54 \mu\text{M}\) and \(0.8 \pm 0.2 \text{ at } -100 \text{ mV (n = 6)}, \) \(40.3 \pm 8.9 \mu\text{M}\) and \(0.9 \pm 0.2 \text{ at } -80 \text{ mV (n = 6)}, \) and \(13.1 \pm 1.8 \mu\text{M}\) and \(1.1 \pm 0.3 \text{ at } -60 \text{ mV (n = 6)}, \) respectively.

**Amlodipine Effect**

- **L-type**
  - Concentration-response curves for amlodipine at holding potentials of \(-100 (○), -80 (■), \) and \(-60 \text{ mV (▲)}\). The IC\(_{50}\) and n\(_{H}\) for an L-type Ca\(^{2+}\) channel are \(4.2 \pm 3.1 \mu\text{M}\) and \(0.7 \pm 0.1 \text{ at } -80 \text{ mV (n = 12)}, \) and \(1.2 \pm 0.6 \mu\text{M}\) and \(1.0 \pm 0.2 \text{ at } -60 \text{ mV (n = 11)}, \) respectively. Those for an N-type Ca\(^{2+}\) channel are \(7.9 \pm 2.6 \mu\text{M}\) and \(0.7 \pm 0.2 \text{ at } -100 \text{ mV (n = 14)}, \) \(1.9 \pm 0.8 \mu\text{M}\) and \(0.7 \pm 0.1 \text{ at } -80 \text{ mV (n = 12)}, \) and \(0.14 \pm 0.05 \mu\text{M}\) and \(0.5 \pm 0.1 \text{ at } -60 \text{ mV (n = 8)}, \) respectively. Those for a P/Q-type Ca\(^{2+}\) channel are \(11.5 \pm 2.4 \mu\text{M}\) and \(1.0 \pm 0.2 \text{ at } -100 \text{ mV (n = 7)}, \) \(7.3 \pm 1.4 \mu\text{M}\) and \(0.8 \pm 0.1 \text{ at } -80 \text{ mV (n = 7)}, \) and \(3.0 \pm 0.9 \mu\text{M}\) and \(0.8 \pm 0.2 \text{ at } -60 \text{ mV (n = 6)}, \) respectively.

To compare the selectivity of blocking action of DHPs on L-, N-, and P/Q-type Ca\(^{2+}\) channels, the IC\(_{50}\) values for these three Ca\(^{2+}\) channels are summarized in Fig. 10. Because voltage dependence of the blocking action on a channel subtype was different in each DHP, we could not simply compare the selectivities of DHPs in blocking Ca\(^{2+}\) channel subtypes. At the holding potential of \(-80 \text{ mV}, \) the rank order of the IC\(_{50}\) values for an N-type Ca\(^{2+}\) channel was amloidipine < cilnidipine < benidipine < barnidipine < nicardipine. The ranking was not changed except the order of benidipine and nicardipine at the holding potential of \(-60 \text{ mV}. \) The IC\(_{50}\) values for amlodipine effect on a P/Q-type Ca\(^{2+}\) channel were smaller compared with the other DHP compounds. The IC\(_{50}\) for nicardipine effect for a P/Q type Ca\(^{2+}\) channel was considerably smaller than that for an N-type Ca\(^{2+}\) channel. The other three DHPs (benidipine, cilnidipine, and barnidipine) showed similar IC\(_{50}\) values for both N- and P/Q-type Ca\(^{2+}\) channels. The IC\(_{50}\) values were not correlated with molecular weight, solubility, or acid dissociation constant (pK\(_a\)) value for DHPs (Table 1). The IC\(_{50}\) values for the effects of these five DHPs on an L-type Ca\(^{2+}\) channel stayed in a narrow range compared with those for N- and P/Q-type Ca\(^{2+}\) channels at the holding potentials of \(-80 \text{ and } -60 \text{ mV.} \) Thus, amlodipine blocked all three Ca\(^{2+}\) channel subtypes with comparable potency, and nicardipine had lower affinity to N-type Ca\(^{2+}\) channels compared with the other four DHPs (benidipine, cilnidipine, barnidipine and amlodipine). Benidipine, cilnidipine, and barnidipine showed similar selectivities for N- and P/Q-type Ca\(^{2+}\) channels.
Discussion

In the present study, five DHP derivatives (amlodipine, benidipine, cilnidipine, nicardipine, and barnidipine) differentially blocked L-, N-, and P/Q-type Ca\textsuperscript{2+} channels that were expressed functionally in Xenopus oocytes, an in vivo expression system. These findings indicate that some DHPs are not selective antagonists for L-type channels. Although our data covered only narrow and hyperpolarized membrane potentials, amlodipine showed high affinity for both N- and P/Q-type channels, and nicardipine preferentially antagonized the P/Q-type channel. Moreover, blockades by benidipine and cilnidipine were voltage-dependent in N-type channels but not in P/Q-type channels. However, further systematic studies are necessary to clarify the channel subtype selectivity of the DHPs because the voltage-dependent effects of the five DHPs on L-type Ca\textsuperscript{2+} channels were different and complex. These findings are consistent with the observations of blocking of N-type channels by cilnidipine in native tissues, dorsal root ganglion neurons (Fujii et al., 1997), and sympathetic neurons (Uneyama et al., 1997). The other five DHPs (nifedipine, nilvadipine, nimodipine, nitrendipine, and felodipine) were strongly selective for L-type channels. Thus, we were able to categorize five DHPs (nifedipine, nilvadipine, nimodipine, nitrendipine, and felodipine) as L-type-selective DHPs and the other five DHPs (amlodipine, benidipine, cilnidipine, nicardipine, and barnidipine) as L-type-nonselective DHPs. These categorizations indicate the importance of determining which DHPs block which subtypes of Ca\textsuperscript{2+} channels for the therapeutic application of these drugs.

In this study, we investigated the DHP effect on the Ca\textsuperscript{2+} channels with the same combination of auxiliary Ca\textsuperscript{2+} channel subunits. Although the \(\alpha_2\) and \(\beta\) subunit combination is known to modulate the DHP action on L-type Ca\textsuperscript{2+} channels.
Ca\textsubscript{2+a} possibly accounted for by the structural differences of the differential blockades of channel subtypes by DHPs are most probably accounted for by the structural differences of the \( \alpha \)1 subunits (Hockerman et al., 1997; Hering et al., 1998; Striessnig et al., 1998). Nine amino acid residues in the subunits (Hockerman et al., 1997; Hering et al., 1998; Welling et al., 1999) were shown that the effect of amlodipine on N-type channels was not substantially modulated by exchanging the \( \beta \)2\textsubscript{A} subunit class (\( \beta \)2\textsubscript{A}, \( \beta \)2\textsubscript{B}, or \( \beta \)2\textsubscript{C}). Therefore, these differential blockades of channel subtypes by DHPs are most probably accounted for by the structural differences of the \( \alpha \)1 subunits (Hockerman et al., 1997; Hering et al., 1998; Striessnig et al., 1998). Nine amino acid residues in the segments IIIS5, IIIS6, and IVS6 of the \( \alpha \)1C or \( \alpha \)1A subunit of L-type Ca\textsuperscript{2+} channels have been shown to interact directly with DHPs (Hockerman et al., 1997; Hering et al., 1998; Striessnig et al., 1998). Eight of these amino acid residues are conserved in the \( \alpha \)1 subunits of non-L-type Ca\textsuperscript{2+} channels, including N-, P/Q-, and R-type channels, and the incorporation of these amino acid sequences made P/Q-type (Sinnegger et al., 1997) and R-type Ca\textsuperscript{2+} channels (Ito et al., 1997) sensitive to DHPs. Taken together with our results that five DHPs (amlodipine, benidipine, cilnidipine, nicardipine, and barnidipine) were able to block N- and P/Q-type channels, these suggest that N- and P/Q-type channels are endowed with a basic structure designed for interaction with the five DHPs. However, R-type channels were not appreciably blocked by any of the DHPs examined. Therefore, amino acid residues other than these conserved four residues may play a critical role in DHP selectivity between these non-L-type channels. The five L-type-nonselective DHPs had larger molecular weights compared with the other DHPs used in this study. Nevertheless, the blocking potency of DHPs on non-L-type channels was not correlated with the structural characteristics of the DHPs, including the degree of ionization (Kass and Arena, 1989) and partition coefficients (Table 1). Further studies using additional DHPs and site-directed mutagenesis on the \( \alpha \)1B and \( \alpha \)1A subunits will be necessary to determine the direct interaction between specific amino acid residues on the \( \alpha \)1 subunits and specific DHPs.

This is the first report that P/Q-type Ca\textsuperscript{2+} channels, as well as N-type channels, were blocked by DHPs with a voltage dependence. DHPs are known to show a voltage- and state-dependent action on L-type Ca\textsuperscript{2+} channels (Hockerman et al., 1997; Striessnig et al., 1998). DHP antagonists bind to the inactivated state of the Ca\textsuperscript{2+} channel with the highest affinity, resulting in a blockade that is steeply voltage dependent. Cilnidipine and benidipine showed a weak voltage dependence. Cilnidipine and benidipine showed a weak voltage dependence.

### Fig. 9
Concentration-response curves for cilnidipine at holding potentials of \(-100\) (●), \(80\) (■), and \(60\) mV (▲). The IC\textsubscript{50} and \( n_{H} \) for an L-type Ca\textsuperscript{2+} channel are 5.3 ± 2.1 \( \mu \)M and 0.2 ± 0.1 at 80 mV (\( n = 10 \)) and 12.7 ± 5.8 \( \mu \)M and 0.9 ± 0.4 at 60 mV (\( n = 9 \)). Those for an N-type Ca\textsuperscript{2+} channel are 39.4 ± 7.9 \( \mu \)M and 0.8 ± 0.2 at 100 mV (\( n = 6 \)), 18.8 ± 3.2 \( \mu \)M and 0.7 ± 0.2 at 80 mV (\( n = 6 \)), and 4.2 ± 1.7 \( \mu \)M and 0.5 ± 0.1 at 60 mV (\( n = 6 \)), respectively. Those for a P/Q-type Ca\textsuperscript{2+} channel are 22.6 ± 7.3 \( \mu \)M and 0.5 ± 0.1 at 100 mV (\( n = 6 \)), 58.5 ± 9.0 \( \mu \)M and 0.4 ± 0.1 at 80 mV (\( n = 6 \)), and 20.8 ± 7.4 \( \mu \)M and 0.4 ± 0.1 at 60 mV (\( n = 6 \)), respectively.

### Fig. 10
Comparison of IC\textsubscript{50} values for the effect of five DHP derivatives on N-, P/Q-, and L-type Ca\textsuperscript{2+} channels. IC\textsubscript{50} values for three Ca\textsuperscript{2+} channel subtypes are plotted against the same semilogarithmic concentration axis.
DHPs such as amlodipine (Burges et al., 1989), barnidipine (van Zwieteren, 1998), bendipine (Fuji et al., 1988), cilnidipine (Minami et al., 1998), and nicardipine (Chen et al., 1995) have been shown not to increase heart rate significantly. However, this DHP effect on heart rate may be closely related to the pharmacokinetics of DHPs because felodipine, one of the L-type selective DHPs, does not increase heart rate (Bicchi et al., 1998).

Three DHPs—amlodipine (Abernetthy et al., 1988), barnidipine (Argenziano et al., 1998), and cilnidipine (Minami et al., 1998)—are also known to decrease the serum catecholamine level. In addition, it has been reported that amlodipine and cilnidipine reduce sympathetic tones as measured by heart rate variability (Hamada et al., 1998; Minami et al., 1998a, b).

To our knowledge, these kinds of sympathetic reactions have not been reported for L-type-selective DHPs. The blocking action of these DHPs on N-type channels may be related to the characteristics of autonomic modulation. This is consistent with the fact that analgesic effects have been shown in o-conotoxin (Miljanich and Ramachandran, 1995), a peptide blocker of N-type channels, as well as amlodipine (Dogrul et al., 1997), which blocked N-type channels most profoundly among the L-type-nonselective DHPs. In addition, the L-type-selective antagonists, such as nifedipine, verapamil, and diltiazem, are considerably less potent in inducing antinociception (Miljanich and Ramachandran, 1995).

P/Q-type channel malfunction recently has been implicated in the pathophysiology of certain forms of migraine, ataxia, and epilepsy (Doyle and Stubbs, 1998; Ophoff et al., 1998). Although the in vivo effects of DHP action on P/Q-type Ca2+ channels are totally unknown, these Ca2+ channels represent therapeutic targets for novel drugs. For this reason, the major challenge for Ca2+ channel pharmacologists is to develop selective nonpeptide modulators of certain non-L-type channels. The findings in this study would provide a new insight into the classification of DHPs based on the selectivity in Ca2+ channel subtypes, as well as helpful information for determining selective DHPs for every non-L-type channel.

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