Actions of Ca\(^{2+}\) Antagonists on Two Types of Ca\(^{2+}\) Channels in Rat Aorta Smooth Muscle Cells in Primary Culture

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Norio Akaike, and Motoomi Nakamura

Mechanisms of blockade of two types of Ca\(^{2+}\) channels by the organic Ca\(^{2+}\) antagonists, nicardipine, diltiazem, verapamil, and flunarizine, were examined in rat aorta smooth muscle cells in primary culture by using the whole-cell voltage-clamp method. T-type Ca\(^{2+}\) current (T-type \(I_{\text{Ca}}\)) was isolated by an internal perfusion of 5 mM F\(^{−}\), which irreversibly suppressed the L-type \(I_{\text{Ca}}\), without affecting T-type \(I_{\text{Ca}}\). L-type \(I_{\text{Ca}}\) was isolated by setting a holding potential at \(-60\) mV, at which most of the T-type Ca\(^{2+}\) channels were inactivated. L-type \(I_{\text{Ca}}\) is halved by 0.1 \(\mu\)M nicardipine, 3.0 \(\mu\)M diltiazem, 0.6 \(\mu\)M verapamil, and 0.1 \(\mu\)M flunarizine, whereas T-type \(I_{\text{Ca}}\) is halved by the same drugs at 0.6, 30, 30, and 0.1 \(\mu\)M, respectively. Diltiazem and verapamil accelerated the decay of L-type \(I_{\text{Ca}}\) and cumulatively blocked L-type \(I_{\text{Ca}}\) during repetitive step depolarizations elicited every 30 seconds (“use-dependent block”). Diltiazem and verapamil neither changed the decay of T-type \(I_{\text{Ca}}\) nor showed a use-dependent block of T-type \(I_{\text{Ca}}\). Nicardipine and flunarizine blocked both L- and T-type \(I_{\text{Ca}}\) from the first depolarization step after drug treatment (“ionic block”) and shifted their steady-state inactivation curves to the left. The estimated binding constants of nicardipine and flunarizine for the inactivated state of T-type Ca\(^{2+}\) channels (48 and 19 nM, respectively) were smaller than those for the resting state of L-type Ca\(^{2+}\) channels (160 and 90 nM, respectively). A low concentration (0.1 \(\mu\)M) of nicardipine initially potentiated T-type \(I_{\text{Ca}}\) and then reduced it. We conclude from these results that 1) nicardipine and flunarizine block not only the resting state but, more preferentially, the inactivated state of both the L- and T-type Ca\(^{2+}\) channels; 2) verapamil and diltiazem preferentially act on the open state of the L-type Ca\(^{2+}\) channel and on the resting and inactivated state of the T-type Ca\(^{2+}\) channel; and 3) the T-type Ca\(^{2+}\) channel of the rat aorta smooth muscle cells appears to be more sensitive to nicardipine and flunarizine than does the L-type Ca\(^{2+}\) channel at around the resting membrane potential. (Circulation Research 1990;67:469–480)

There is reported evidence of two types of Ca\(^{2+}\) channels in vascular smooth muscle cells (SMCs)\(^1\)-\(^3\); one is an L-type Ca\(^{2+}\) channel, and the other is a T-type Ca\(^{2+}\) channel. Although the T-type Ca\(^{2+}\) current is relatively insensitive to Ca\(^{2+}\) antagonists,\(^1\)-\(^7\) we did observe that the T-type Ca\(^{2+}\) channel of cultured SMCs of the rat aorta is also sensitive to Ca\(^{2+}\) antagonists such as flunarizine and nicardipine.\(^8\) Mechanisms related to actions of organic Ca\(^{2+}\) antagonists, even on the L-type Ca\(^{2+}\) channel in vascular SMCs, are not well understood. Most studies were done under conditions in which both types of Ca\(^{2+}\) currents may coexist, as based on the assumption that the T-type Ca\(^{2+}\) channel is insensitive to organic Ca\(^{2+}\) antagonists.\(^1\)-\(^7\) Thus, to better comprehend precise mechanisms of actions of the Ca\(^{2+}\) antagonists on the two types of Ca\(^{2+}\) channels of vascular SMCs, each Ca\(^{2+}\) current had to be isolated.

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We used the whole-cell patch-clamp method$^9$ and attempted to elucidate mechanisms of action of four representative types of Ca$^{2+}$ antagonists on the two types of Ca$^{2+}$ channels in rat aorta SMCs in primary culture. With this preparation, each type of the Ca$^{2+}$ current could be readily isolated, and the amplitude of the current was large enough for a quantitative analysis. We found that the effects of Ca$^{2+}$ antagonists on the two types of Ca$^{2+}$ channels of vascular SMCs were state dependent. Nicardipine and flunarizine had a voltage-dependent effect on both types of Ca$^{2+}$ channels. Because more than half of the T-type Ca$^{2+}$ channel may be inactivated around the resting membrane potential, the T-type Ca$^{2+}$ channel of the rat aorta seems to be more sensitive to nicardipine and flunarizine than does the L-type Ca$^{2+}$ channel at around the resting membrane potential.

Materials and Methods

Preparations

Male Wistar rats (250–300 g) were given on overdose of ether. SMCs of the aortic media were dispersed enzymatically and grown in primary culture, as described previously.$^{10}$ Using quin 2 microfluorometry, we found that diltiazem and verapamil dose dependently inhibited elevation of the cytosolic Ca$^{2+}$ induced by high external K$^+$ in the same preparation.$^{11}$ Experiments were performed on cells at 5–15 days of culture. The direct immunofluorescence staining of smooth muscle myosin and actin excluded the contamination of fibroblasts.$^{12}$ Harder and Sperelakis$^{13}$ reported that the resting membrane potential of cultured SMCs of the rat aorta was −55 mV.

Electrical Measurements

The cultured cells on the coverslips (No. 5410, Thermaxo, Naperville, Ill.) were trypsinized for 1 minute, placed in a recording chamber, and continuously superfused with an external solution at a rate of 1 ml/min. Heat-polished glass patch electrodes (VC-H075P, Terumo, Tokyo, Japan) with a tip resistance of about 2–5 MΩ were used. We used the whole-cell voltage-clamp method.$^9$ The currents were amplified with a patch-clamp amplifier (EPC-7, List-Electronic, Darmstadt-Eberstadt, FRG) with capacitance and series-resistance compensation, filtered at 2.0 kHz, and analyzed with an on-line computer (PC-9801 XL, NEC, Tokyo, Japan). All experiments were monitored on a digital storage scope (DS-6121A, Iwatsu, Tokyo, Japan) and were simultaneously stored on a PCM data recorder (RP-880, NF Circuit Design Block, Yokohama, Japan) for later analyses. The amplitude of the inward current was measured at the peak of each current. Leakage and capacitative currents were subtracted by using currents elicited by the small hyperpolarizing pulse.

Solutions

The external solution contained (mM) CaCl$_2$ 20, NaCl 110, KCl 2, HEPES 5, glucose 10, tetraethylammonium chloride (TEA-Cl) 15, and 4-aminopyridine 5. The pH was adjusted to 7.3 with Tris(hydroxymethyl)aminoethane hydroxide. The internal solution contained (mM) N-methyl-d-glucamine (NMG) 110, Na$_2$ATP 5, MgSO$_4$ 5, TEA-Cl 20, HEPES 5, Tris(hydroxymethyl)aminoethane hydroxide 2, EGTA 10, adenosine 3′,5′-cyclic monophosphate (cAMP) 1. When investigating T-type Ca$^{2+}$ current (T-type I$_{ca}$), we added 5 mM hydrofluoric acid to the internal solution to suppress L-type I$_{ca}$. The pH of the internal solution was adjusted to 7.2 with HCl. The exchange of the external solution was accomplished within 60 seconds. All experiments were carried out at room temperature (22–24°C).

Characterization and Separation of the Two Types of Ca$^{2+}$ Currents

Electrical and pharmacological properties of the L- and T-type I$_{ca}$ of the SMCs of the rat aorta in primary culture were identical to those described previously.$^8$ In brief, L-type I$_{ca}$ was activated by step depolarizations to potentials more positive than −20 mV and reached a peak around 20 mV from a holding potential (V$_{H}$) of −60 mV, while T-type I$_{ca}$ was activated at around −60 mV and reached a peak at −30 mV from a V$_{H}$ of −100 mV. Neither 60 μM tetrodotoxin nor substitution of extracellular Na$^+$ by Tris$^+$ decreased L- or T-type I$_{ca}$. Both currents were increased by increasing the extracellular Ca$^{2+}$ concentrations. In cells cultured for 6–10 days, many types of populations were observed; cells having mostly the L-type Ca$^{2+}$ channel, those with mostly the T-type Ca$^{2+}$ channel, and those with both types of Ca$^{2+}$ channels, in various proportions. When investigating T-type I$_{ca}$, we used cells that seemed to have only the T-type I$_{ca}$. In addition, an internal dialysis of F$^−$ was used to completely inactivate L-type I$_{ca}$, affecting neither T-type I$_{ca}$ itself nor actions of the Ca$^{2+}$ antagonists on the T-type I$_{ca}$. When investigating L-type I$_{ca}$, we used cells that seemed to have only L-type I$_{ca}$. We inactivated T-type I$_{ca}$ by setting the V$_{H}$ at −60 mV, a point where the L-type Ca$^{2+}$ channel is little inactivated.$^8$ Cells with a current amplitude of above 0.1 nA were used in all experiments. We conducted the same series of experiments on freshly isolated aorta SMCs. Electrophysiological and pharmacological characteristics of both types of the Ca$^{2+}$ channels were qualitatively similar in freshly dispersed and primary cultured cells; therefore, we used primary cultured cells in the present study.

Drugs

The drugs used were trypsin (GIBCO Laboratories, Grand Island, N.Y.), hydrofluoric acid (Daikin Kogyo, Osaka, Japan), nicardipine (Yamanouchi, Tokyo, Japan), diltiazem (Tanabe, Osaka, Japan), verapamil (Eisai, Tokyo, Japan), flunarizine (Kyowa Hakko, Tokyo, Japan), and NMG (Tokyo Kasei, Tokyo, Japan). All drugs were prepared as a stock solution and were made to a final concentration with external solution. Nicardipine was dissolved in...
Effects of Ca\(^{2+}\) antagonists on current-voltage (I-V) relations of L- and T-type Ca\(^{2+}\) current (I\(_{Ca}\)). Panel A: L-type I\(_{Ca}\) was evoked by various step depolarizations of 300 msec from a holding potential (V\(_{H}\)) of −60 mV in the absence (○) and presence (●) of the Ca\(^{2+}\) antagonist in the same cell. Panel B: T-type I\(_{Ca}\) was evoked by step depolarizations having 300 msec duration from a V\(_{H}\) of −100 mV in the absence (○) and the presence (●) of the drug in the same cell. The amplitude of I\(_{Ca}\) in the presence of the Ca\(^{2+}\) antagonists was measured when the maximum inhibition was attained by repetitive applications of the command pulse at 30-second intervals. The insets show the peak currents in the absence (○) and the presence (●) of each Ca\(^{2+}\) antagonist evoked by the test potentials, shown by the arrowheads on the individual I-V relations. [Ca\(^{2+}\)]\(_0\) was 20 mM.

99% ethanol to make a 1 mM stock solution and was protected from exposure to light. When this concentration was used, ethanol had no effects on either L- or T-type I\(_{Ca}\).

Statistics

Data are given as the mean±SEM (n=number of experiments). Theoretical curves were fitted to the data with the least-squares method. Statistical significance was determined with Student’s t test.

Results

Effects of Ca\(^{2+}\) Antagonists on Current-Voltage Relations

Figure 1 shows the effects of the four representative Ca\(^{2+}\) antagonists, nicardipine, diltiazem, verapamil, and flunarizine, on current-voltage relations of L- and T-type I\(_{Ca}\) of rat aorta SMCs in primary culture. The concentration of each Ca\(^{2+}\) antagonist used in these experiments was near the 50% inhibition concentration (IC\(_{50}\)) required to inhibit the Ca\(^{2+}\) currents. Insets show the original tracings of the inhibitions at the peak on the current-voltage relations. The amplitude of Ca\(^{2+}\) current in the presence of the Ca\(^{2+}\) antagonists was evaluated after the inhibition had reached maximum by repetitive applications of step depolarizations at 0.033 Hz. Each Ca\(^{2+}\) antagonist inhibited both L- and T-type I\(_{Ca}\) at all examined test potentials, without significantly altering the current-voltage relations. Similar results were obtained from five additional cells for each Ca\(^{2+}\) antagonist.

Tonic and Use-Dependent Block

To examine the time course of the block induced by the Ca\(^{2+}\) antagonists, we applied step pulses at various frequencies after the drug application. The concentration of each Ca\(^{2+}\) antagonist was selected near the IC\(_{50}\) determined by applying repetitive step pulses at 0.033 Hz. In the absence of the Ca\(^{2+}\) antagonists, the amplitudes of both types of I\(_{Ca}\) were...
stable with repetitive stimulations at 0.5 Hz and normalized as 1.0. After a 3-minute quiescent period, command pulses were initially applied at the rate of 0.033 Hz. When the blocking effect at each frequency became stable, the frequency was elevated to 0.1, 0.2, and 0.5 Hz. When the frequency of the command pulse was 0.033 Hz, blocking effects of nicardipine and flunarizine on L-type $I_{\text{Ca}}$ showed a maximum at the first command pulse, while those of diltiazem and verapamil showed an accumulation of block during repetitive stimulations (Figure 2). In the case of T-type $I_{\text{Ca}}$ at 0.033 Hz, nicardipine, diltiazem, and verapamil showed a maximum block at the first command pulse, while flunarizine showed an accumulation of block (Figure 3). The blocking effect obtained at the first command pulse after drug application was termed “tonic block,” while the block accumulating during repetitive stimulations was termed “use-dependent block.” When the tonic block was evaluated at 3 minutes after drug application, the dominant tonic block of L-type $I_{\text{Ca}}$ was noted in the cases of nicardipine and flunarizine, while that of T-type $I_{\text{Ca}}$ was noted for nicardipine, diltiazem, and verapamil (Tables 1 and 2). When the use-dependent block was observed with step depolarizations of 0.033 Hz, the first step depolarization was applied after an 8-minute pretreatment of the drugs, and the tonic block was also evaluated at 8 minutes in other cells (open circles in Figures 2 and 3). The tonic block of L-type $I_{\text{Ca}}$ by diltiazem and verapamil evaluated at 8 minutes did not significantly differ from that evaluated at 3 minutes. The tonic block of T-type $I_{\text{Ca}}$ by flunarizine evaluated at 8 minutes was, however, significantly more profound than that evaluated at 3 minutes (Figures 2 and 3, Table 2).
The use-dependent block of L-type \( I_{\text{Ca}} \) by diltiazem and verapamil was noted with stimulations at rates higher than 0.033 Hz and that by flunarizine appeared at 0.5 Hz. In the case of nicardipine, the use-dependent block of L-type \( I_{\text{Ca}} \) did not appear even at 0.5 Hz. When the concentration of flunarizine was elevated, the use-dependent block of L-type \( I_{\text{Ca}} \) appeared at lower than 0.5 Hz (not shown). The use-dependent block of T-type \( I_{\text{Ca}} \) by nicardipine, diltiazem, verapamil, and flunarizine appeared at 0.5, 0.2, 0.2, and 0.033 Hz, respectively. When the blocking was used dependent, the blocking level reached a steady state at each frequency of stimulation. The blocking level became more profound as the frequency of the stimulation was elevated (Figures 2 and 3).

When the inhibition showed evidence of use dependency, command pulses were temporarily

| Table 1. Tonic and Use-Dependent Block of L-Type Ca\(^{2+}\) Current |
|-----------------------------|-----------------------------|-----------------------------|
|                             | Tonic block (%)             | Use-dependent block (%)      |
|                             | 3 min                       | 8 min                       | 0.033 Hz | 0.5 Hz |
| Nicardipine (0.1 \( \mu \text{M} \)) | 53±6                        | ND                          | 53±6     | 47±6   |
| Diltiazem (3.0 \( \mu \text{M} \)) | 87±2                        | 85±5                        | 57±8*    | 22±4*  |
| Verapamil (0.6 \( \mu \text{M} \)) | 90±4                        | 88±6                        | 51±4*    | 10±2*  |
| Flunarizine (0.1 \( \mu \text{M} \)) | 56±2                        | ND                          | 50±25    | 39±5*  |

Values are mean±SEM of six experiments expressed as a percentage of zero time control data. The tonic block means the block observed at the first command pulse after drug application. The use-dependent block means the block attained by repetitive stimulations. ND, not determined.

*Significantly \((p<0.01)\) different compared with the tonic block evaluated at 3 minutes of the respective drugs.
halted in the presence of the drugs, and after a 3-minute quiescent period, the command pulses were resumed. The recovery of L-type ICa from inhibition by verapamil and diltiazem was observed during a 3-minute quiescent period, while recovery of T-type ICa from inhibition by flunarizine was slight.

After washing out each Ca\textsuperscript{2+} antagonist, recovery from the inhibition of both types of ICa by nicardipine, diltiazem, and verapamil was almost complete within 10 minutes. However, the inhibitory effect of flunarizine was irreversible (Figures 2 and 3).

**Potentiation of T-Type Ca\textsuperscript{2+} Current by Low Concentrations of Nicardipine**

Figure 4 shows the time course of effects of a low concentration of nicardipine (0.1 \(\mu\)M) on the amplitude of T-type ICa evoked every 30 seconds. Nicardipine (0.1 \(\mu\)M) initially increased the amplitude of T-type ICa for a few minutes and then reduced it. The stimulatory effects became less obvious, and only inhibitory effects were observed when a higher concentration of nicardipine was used. Nicardipine at any concentration used did not potentiate L-type ICa. The other Ca\textsuperscript{2+} antagonists used showed no such stimulatory effects on either type of ICa.

**Effects of Ca\textsuperscript{2+} Antagonists on the Decay of Ca\textsuperscript{2+} Current**

Effects of the Ca\textsuperscript{2+} antagonists on the decay of the Ca\textsuperscript{2+} currents were then examined. In the absence of the drugs, the decay of the L-type ICa was fitted by a sum of two exponential functions.\textsuperscript{8} Although the time constant for the slower phase (\(\tau_2\)) remained the same, the time constant for the faster phase (\(\tau_1\)) was prolonged during the experiments (Figure 5A). These phenomena were noted also in Ca\textsuperscript{2+} currents of the Helix neuron and were attributed to the intracellular perfusion of EGTA.\textsuperscript{16} The decay of T-type ICa was fitted by a single exponential function in which the time constant remained steady for 30 minutes.\textsuperscript{8} Figures 5B and 5C show the effects of verapamil and diltiazem on the decay of L-type ICa evoked by the first application of command pulse after an 8-minute treatment of the drugs. Compared with the control tracings in Figure 5A, verapamil and diltiazem slightly accelerated both phases of decay of L-type ICa. Nicardipine and flunarizine, however, did not alter the decay of L-type ICa (not shown). None of the Ca\textsuperscript{2+} antagonists used had any apparent effect on the decay of T-type ICa (not shown).

**Voltage-Dependent Block**

Voltage-dependent blocks were examined quantitatively by using the double-pulse protocol. The \(V_H\) was set at -80 mV for L-type ICa and at -120 mV for T-type ICa. The duration of prepulse was 3 seconds, a time sufficient to attain a steady-state inactivation of both types of ICa.\textsuperscript{8} The double-pulse method in the presence of each Ca\textsuperscript{2+} antagonist was carried out after the inhibition at each \(V_H\) had reached a steady state by repetitive stimulations at 0.033 Hz. In the experiment shown in Figure 6, the blocking effect of nicardipine on L-type ICa increased as the prepulse potential depolarized. Figures 7A and 8A show relations between the prepulse potential and the amplitude of ICa, and Figures 7B and 8B show the steady-state inactivation curves obtained by normalizing the current evoked from a \(V_H\) without inactivation as 1.0. Continuous lines were fitted by the Boltzmann equation using the least-squares method. Table 3 summarizes the effects of the Ca\textsuperscript{2+} antagonists on the parameters of this equation. Nicardipine, diltiazem, and flunarizine significantly shifted the steady-state inactivation curve of L-type ICa toward the negative potential in the order of nicardipine > flunarizine > diltiazem, while verapamil slightly affected the

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**Table 2. Tonic and Use-Dependent Block of T-Type Ca\textsuperscript{2+} Current**

<table>
<thead>
<tr>
<th></th>
<th>Tonic block</th>
<th>Use-dependent block</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 min</td>
<td>8 min</td>
</tr>
<tr>
<td>Nicardipine (0.6 (\mu)M)</td>
<td>64±3</td>
<td>ND</td>
</tr>
<tr>
<td>Diltiazem (10 (\mu)M)</td>
<td>60±5</td>
<td>ND</td>
</tr>
<tr>
<td>Verapamil (30 (\mu)M)</td>
<td>61±4</td>
<td>ND</td>
</tr>
<tr>
<td>Flunarizine (0.1 (\mu)M)</td>
<td>83±3</td>
<td>72±5*</td>
</tr>
</tbody>
</table>

Values are mean±SEM of six experiments expressed as a percentage of zero time control data. ND, not determined.

*Significantly (p<0.01) different compared with the tonic block evaluated at 3 minutes of the respective drugs.
steady-state inactivation curve (n=5). Nicardipine and flunarizine, but not diltiazem and verapamil, significantly shifted the steady-state inactivation curve of T-type ICa to the left (n=5).

**Discussion**

Since the preparation we used had the definite advantage in that each of the two types of the ICa could be isolated, the effects of the Ca\(^{2+}\) antagonists

FIGURE 5. Changes in the decay of L-type Ca\(^{2+}\) current (ICa). L-type ICa was activated by command pulses to 20 mV from a holding potential (VH) of -60 mV. The first command pulse was applied in the absence of the drugs, and the second pulse was applied after 8 minutes in the absence (panel A) and presence (panels B and C) of the drugs that had been applied just after the first pulse. Effect of Ca\(^{2+}\) antagonists on the open channel was best observed in the first test pulse after drug application, as described by Lee and Tsien.\(^{15}\) [Ca\(^{2+}\)]\(_o\) was 20 mM. Semilogarithmic plots of the inactivation time course of L-type ICa elicited by the first and the second step pulses are shown. Inactivation of the L-type ICa was fitted by a sum of double-exponential functions. Each graph shows both fast and slow components of the inactivation. We named the time constant for the fast falling phase (\(\tau_{h2}\)) and that for the slow falling phase (\(\tau_{h1}\)). Insets show actual records of L-type ICa elicited by the first (○) and the second (●) step pulses. Panel A: In the absence of the Ca\(^{2+}\) antagonists, \(\tau_{h1}\) and \(\tau_{h2}\) were 38 and 470 msec, respectively, at first and were 62 and 480 msec, respectively, at 8 minutes. Panel B: Effects of verapamil (0.6 μM) on the inactivation of L-type ICa. \(\tau_{h1}\) and \(\tau_{h2}\) decreased from 48 to 38 and from 426 to 325 msec, respectively, in the presence of verapamil. Panel C: Effects of diltiazem (3.0 μM) on the inactivation of L-type ICa. \(\tau_{h1}\) values were 44 and 43 msec before and after adding diltiazem. \(\tau_{h2}\) values were 545 and 415 msec before and after the addition of diltiazem.
on the L- and T-type Ca\(^{2+}\) channels of vascular SMCs could be evaluated independently. The four representative Ca\(^{2+}\) antagonists showed different effects on the two types of Ca\(^{2+}\) channels in vascular SMCs of the rat aorta in primary culture, according to the modulated receptor theory,\(^{14,15,17}\) in which it is postulated that the affinity of a drug for a binding site is modulated by channel gating. Nicardipine and flunarizine acted on not only the resting state but more preferentially the inactivated state of both the L- and T-type Ca\(^{2+}\) channels. While verapamil and diltiazem preferentially acted on the open state of the L-type Ca\(^{2+}\) channel, these drugs acted on the resting and the inactivated state of the T-type Ca\(^{2+}\) channel.

**Tonic Block**

As the tonic block was evaluated at the holding potential of −60 mV for L-type ICa and −100 mV for T-type ICa, the observed inhibition shows the effects on the state without inactivation (resting state).\(^{8}\) Our results suggest that nicardipine and flunarizine act on the resting state of L-type Ca\(^{2+}\) channel in SMCs of the rat aorta. The effects of dihydropyridine and diphenylalkylamine on the resting state of the L-type Ca\(^{2+}\) channel have been noted in frog atrial cells (nifedipine\(^{14}\)) and in SMCs of rabbit small intestine (nicardipine\(^{18}\) and flunarizine\(^{19}\)). Because the resting potential of the vascular SMCs is around −50 to −70 mV,\(^{13,20}\) at which most of the L-type Ca\(^{2+}\) channels are in the resting state, effects of Ca\(^{2+}\) antagonists on the resting state are critical for the electrically quiescent cell such as the SMC of the rat aorta. Diltiazem and verapamil had little effect on the resting state of the L-type Ca\(^{2+}\) channel. These results suggest that, in the rat aorta SMCs, nicardipine and flunarizine are more effective in blocking the L-type Ca\(^{2+}\) channel than are diltiazem and verapamil, by more than their differences in the IC\(_{50}\)s obtained from the final blocking levels with repetitive stimulations at 0.033 Hz.

In the case of the T-type Ca\(^{2+}\) channel, all drugs examined produced a marked tonic block, results that suggest that the ionizable Ca\(^{2+}\) antagonists such as diltiazem and verapamil preferentially act on the resting state of the T-type Ca\(^{2+}\) channel of cultured rat aorta SMCs.

**Use-Dependent Block**

Verapamil and diltiazem showed a use-dependent block of L-type ICa. Since these drugs accelerated
T-type \( I_{\text{Ca}} \)

For diltiazem, \( V_{0.5} = -71 \) mV and for control, \( V_{0.5} = -70 \) mV and \( k = 6.1 \), while for control, \( V_{0.5} = -71 \) mV and \( k = 6.4 \). For flunarizine, \( V_{0.5} = -77 \) mV and \( k = 7.1 \), while for control \( V_{0.5} = -70 \) mV and \( k = 6.8 \).

All the drugs we examined showed a use-dependent block of T-type \( I_{\text{Ca}} \) when the frequency of the stimulations was increased. Since none of the drugs affected decay of the T-type \( I_{\text{Ca}} \), they did not act on the open state of the T-type Ca\(^{2+}\) channel, yet they might act on the inactivated state and slow the repriming from inactivation. In the absence of the drug, the T-type Ca\(^{2+}\) channel reprimed within 500 msec from the inactivation attained with 300 msec step depolarization to -20 mV (unpublished observations). Nicardipine, diltiazem, verapamil, and flunarizine prolonged the repriming time of the T-type

**Table 3. Effect of Ca\(^{2+}\) Antagonists on Parameters of the Boltzmann Equation**

<table>
<thead>
<tr>
<th>Drug</th>
<th>( I_{\text{max}} ) (pA)</th>
<th>( V_{0.5} ) (mV)</th>
<th>( k )</th>
<th>( I_{\text{max}} ) (pA)</th>
<th>( V_{0.5} ) (mV)</th>
<th>( k )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>184±16</td>
<td>-27±3.2</td>
<td>9.0±0.4</td>
<td>208±17</td>
<td>-64±1.5</td>
<td>6.2±1.0</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>82±17*</td>
<td>-43±4.3*</td>
<td>8.2±0.5</td>
<td>113±8*</td>
<td>-76±2.5*</td>
<td>6.0±0.7</td>
</tr>
<tr>
<td>Control</td>
<td>172±22</td>
<td>-30±2.9</td>
<td>7.5±0.8</td>
<td>194±22</td>
<td>-57±2.5</td>
<td>7.1±1.2</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>86±9*</td>
<td>-36±4.3†</td>
<td>7.7±0.7</td>
<td>125±13*</td>
<td>-61±2.0</td>
<td>7.7±0.8</td>
</tr>
<tr>
<td>Control</td>
<td>180±19</td>
<td>-32±3.0</td>
<td>8.0±0.7</td>
<td>231±56</td>
<td>-66±1.5</td>
<td>7.0±1.0</td>
</tr>
<tr>
<td>Verapamil</td>
<td>86±9*</td>
<td>-34±3.5</td>
<td>8.2±0.7</td>
<td>136±46*</td>
<td>-63±2.6</td>
<td>7.2±0.9</td>
</tr>
<tr>
<td>Control</td>
<td>175±36</td>
<td>-30±3.0</td>
<td>9.0±0.5</td>
<td>213±17</td>
<td>-70±0.5</td>
<td>6.7±0.4</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>88±16*</td>
<td>-41±3.0*</td>
<td>8.7±0.5</td>
<td>131±7*</td>
<td>-84±2.5*</td>
<td>8.3±1.1</td>
</tr>
</tbody>
</table>

Values are mean±SEM of five experiments. The mid-potential \( (V_{0.5}) \) and the slope factor \( (k) \) of steady-state inactivation curves were calculated by the Boltzmann equation using the least-squares method. \( I_{\text{Ca}}, \text{Ca}^{2+}\) current; \( I_{\text{max}} \), the maximum current used to normalize the Boltzmann equation.

*\( p<0.01 \) vs. control.
†\( p<0.05 \) vs. control.
### Table 4. Estimated Equilibrium Constants for Binding of Nicardipine and Flunarizine to Resting and Inactivated States of L- and T-Type Ca\(^{2+}\) Channels

<table>
<thead>
<tr>
<th></th>
<th>L-type</th>
<th>T-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(K_h) (nM)</td>
<td>(K_i) (nM)</td>
</tr>
<tr>
<td>Nicardipine</td>
<td>160</td>
<td>9</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>90</td>
<td>11</td>
</tr>
</tbody>
</table>

According to the modulated receptor hypothesis with 1:1 binding to the resting state and inactivated state of L- and T-type Ca\(^{2+}\) channels (IC\(_a\)), the equilibrium constants for binding to the resting state (\(K_h\)) can be estimated from the block of current at holding potential of \(-80\) mV for L-type IC\(_a\) and \(-120\) mV for T-type IC\(_a\) using the following equation: \(I(\text{drug})/I(\text{control}) = 1/(1+[\text{drug}]/[K_h])\), where \(I(\text{drug})\) means the mean current amplitude in the presence of nicardipine or flunarizine, obtained from the data in Figures 7 and 8. The equilibrium constants for binding to inactivated channels (\(K_i\)) can be estimated from the shift of the midpoint of the steady-state inactivation curve (\(V_i\)) using the following equation: \(V_i = k \ln(1+[\text{drug}]/[K_h])/(1+[\text{drug}]/[K_h])\), where \(k\) is the slope factor of the steady-state inactivation curve fitted by the Boltzmann equation.

Ca\(^{2+}\) channel beyond 2, 5, 5, and 30 seconds, respectively. In the T-type Ca\(^{2+}\) channel, a longer depolarization only not prolonged the repriming time but also changed the time course from a single exponential function to a sum of more than double exponential functions, thereby suggesting the existence of a further inactivated state from which recovery required a long time. The Ca\(^{2+}\) antagonists might absorb the T-type Ca\(^{2+}\) channel into such a profound inactivated state.

The accumulation of block of T-type IC\(_a\) by flunarizine observed at the stimulation frequency of 0.033 Hz might relate not only to the slowing effects on the repriming but also to a slow action on the resting state of the T-type Ca\(^{2+}\) channel, because the tonic block evaluated at 8 minutes was more profound than that seen at 3 minutes. These slow actions of flunarizine on the L-type Ca\(^{2+}\) channel were noted in SMCs of the rabbit small intestine.

**Stimulatory Effect of Nicardipine on T-Type Ca\(^{2+}\) Current**

For the first few minutes of application, a low concentration of nicardipine augmented the T-type IC\(_a\). Such an agonistic effect of dihydropyridines on the L-type IC\(_a\) has been noted in cardiac myocytes. The augmentation of T-type IC\(_a\) by nicardipine was observed only at low concentrations in the present experiments, a finding differing from the effect of nitrrendipine on L-type Ca\(^{2+}\) channels in cardiac myocytes in which the augmentation was dose dependent and was seen even at high concentrations. The augmentation was not affected by the rate of intracellular perfusion. The effect of nicardipine was not due to suppression of the outward current nor to progression of the intracellular dialysis (which suppressed outward currents), since such effects were observed reproducibly only with the following combination: T-type IC\(_a\) and a low concentration of nicardipine.

### Table 5. Chemical Characteristics of Ca\(^{2+}\) Antagonists

<table>
<thead>
<tr>
<th></th>
<th>MW</th>
<th>(pK_a)</th>
<th>Ionization at pH 7.3 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nicardipine</td>
<td>388,425</td>
<td>7.0</td>
<td>33</td>
</tr>
<tr>
<td>Flunarizine</td>
<td>406,50</td>
<td>7.6</td>
<td>67</td>
</tr>
<tr>
<td>Diltiazem</td>
<td>414.52</td>
<td>7.7</td>
<td>71</td>
</tr>
<tr>
<td>Verapamil</td>
<td>454.54</td>
<td>8.7</td>
<td>96</td>
</tr>
</tbody>
</table>

Degree of the ionization was calculated by the Henderson-Hasselbalch equation: \(pK_a-pH=\log ([\text{ionized drug}]/[\text{total drug}])\), where \(pK_a\) is the ionization constant.

Augmentation of the T-type IC\(_a\) might explain effects of the Ca\(^{2+}\) antagonists on inward currents, including both T- and L-type Ca\(^{2+}\) currents in other preparations. The current-voltage relation in the presence of the drugs showed less inhibitory effects in the activation range of the T-type IC\(_a\) than in the more positive membrane potentials.

Since synthetic Ca\(^{2+}\) agonists such as Bay K 86448 or YC 170 (unpublished observations) did not potentiate the T-type IC\(_a\), nicardipine seems to be a unique synthetic Ca\(^{2+}\) antagonist augmenting the T-type IC\(_a\) in SMCs. It has been reported that angiotensin II augments the T-type IC\(_a\) in bovine adrenal glomerulosa cells.

**Voltage Dependency**

In both L- and T-type Ca\(^{2+}\) channels, effects of nicardipine and flunarizine were more prominent when the membrane was held at more depolarized potentials and shifted the steady-state inactivation curves to the left. The results suggest that the drugs bind more strongly to the inactivated state than to the resting state for both types of the Ca\(^{2+}\) channels.

Using the modulated receptor theory, we quantitated the binding constants of nicardipine and flunarizine to the resting and inactivated state of both types of Ca\(^{2+}\) channels. As shown in Table 4, nicardipine acts on the inactivated state with a 17.7 and 14.8 times higher sensitivity than it does on the resting state of L- and T-type Ca\(^{2+}\) channels, respectively. Flunarizine acts on the inactivated state with an 8.2 and 7.2 times higher sensitivity than it does on the resting state of T-type Ca\(^{2+}\) channels. Although the voltage-dependent effects of nicardipine and flunarizine on the L-type Ca\(^{2+}\) channel have been noted in various preparations, such effects for the T-type Ca\(^{2+}\) channel are less documented in the case of bovine adrenal glomerulosa cells, in which nitrrendipine (0.3 \(\mu\)M) shifted the steady-state inactivation curve to the left (8 mV).

Heretofore, the isolation of the T-type IC\(_a\) in some preparations was made by the subtraction method in which a current elicited by a step depolarization from a more hyperpolarized potential such as \(-80\) mV was subtracted by a current elicited by a step depolarization to the same test potential from a more depolarized holding potential such as \(-30\) mV, at which most of the T-type Ca\(^{2+}\) channels were inactivated. Since effects of some organic Ca\(^{2+}\) antagonists...
depend on $V_{th}$, the blockade of the L-type $I_{Ca}$ elicited from the more depolarized holding potential was sometimes larger than that elicited from the more hyperpolarized holding potential. Thus, the effects of the $\text{Ca}^{2+}$ antagonist on the T-type $I_{Ca}$ might have been underestimated.

**T-Type $\text{Ca}^{2+}$ Channel is Sensitive to Nicardipine and Flunarizine**

It has been reported that T-type $I_{Ca}$ of various preparations, including vascular SMCs, is resistant to dihydropyridine $\text{Ca}^{2+}$ antagonists. However, T-type $I_{Ca}$ of the rat aorta SMCs in primary culture is sensitive to nicardipine and flunarizine. Since the resting membrane potential of vascular SMCs is $-50$ to $-70$ mV, more than half of the T-type $\text{Ca}^{2+}$ channels are inactivated around the resting membrane potential in vivo, while the L-type $\text{Ca}^{2+}$ channels are little inactivated under the same condition. Thus, considering the binding constants at the resting state of L-type $I_{Ca}$ with those at the inactivated state of T-type $I_{Ca}$ (Table 4), it is evident that in the resting membrane potential, the T-type $\text{Ca}^{2+}$ channel was more sensitive to both nicardipine and flunarizine than was the L-type $\text{Ca}^{2+}$ channel. This result seems to be important when attempting to interpret the result of experiments in vivo or in vitro. The sensitivity to organic $\text{Ca}^{2+}$ antagonists is high; thus, not only the L-type $\text{Ca}^{2+}$ channel may be responsible for the observed phenomenon.

**Hydrophilic and Hydrophobic Pathways for the $\text{Ca}^{2+}$ Antagonist-Receptor Reaction**

According to the modulated receptor hypothesis, hydrophilic and hydrophobic drugs seem to reach a single receptor associated with the channel via different pathways. The relative contribution of each pathway is determined by the hydrophilicity of the drug; a hydrophilic drug will gain access only to the open channel by way of the channel lumen, while a hydrophobic drug may also have access to the resting and inactivated ion channels by way of plasma lipids. The hydrophilicity of the $\text{Ca}^{2+}$ antagonists depends on the proportion of the ionized form of the drug, which is determined by the difference between the ionization constant ($pK_a$) and pH. Table 5 summarizes parameters concerning the hydrophilicity of the $\text{Ca}^{2+}$ antagonists: $pK_a$ and the degree of the ionization constant at pH 7.3. By increasing the degree of the ionization (hydrophilicity), such as in the case of verapamil, the high accessibility to the open state of the L-type $\text{Ca}^{2+}$ channel became apparent. Conversely, by increasing the proportion of the neutral form (hydrophobicity), such as in the case of nicardipine, accessibility to the resting and especially to the inactivated state of the L-type $\text{Ca}^{2+}$ channel also occurs. These observations suggest that the affinity of each $\text{Ca}^{2+}$ antagonist to the voltage-dependent state of the L-type $\text{Ca}^{2+}$ channel of vascular SMCs is well supported by the modulated receptor hypothesis. Although nicardipine and flunarizine showed specific inhibitory effects on the T-type $\text{Ca}^{2+}$ channel via hydrophilic pathways, diltiazem and verapamil showed unique effects that seem to be independent of the degree of the ionization. While we have no clear explanation as to why verapamil and diltiazem were less effective in suppressing T-type $I_{Ca}$, the sensitivity to and the mode of actions of the $\text{Ca}^{2+}$ antagonists, in the ionized form, might be important differences between L- and T-type $\text{Ca}^{2+}$ channels, in vascular SMCs.

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KEY WORDS • calcium channel • calcium antagonist • whole-cell clamp method • vascular smooth muscle cell
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