State-Dependent Inhibition of L-type Ca^{2+} Channels in A7r5 Cells by Cilnidipine and Its Derivatives

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ABSTRACT—Using a whole-cell patch-clamp technique, state-dependent inhibition of dihydropyridines (DHP)s was investigated on L-type channels in A7r5 cells. Cilnidipine, its derivatives (D-342 and D-69) and nimodipine inhibited the Ba^{2+} current. However, cilnidipine and D-342, but not D-69 or nimodipine, accelerated current decay. The apparent rank order for the effects on the DHP-sensitive decaying component was different from that obtained for inhibition of the peak current. The dissociation constants for cilnidipine in the resting and inactivated states were estimated to be 190 and 12 nM, respectively. Cilnidipine, but not other DHP derivatives, created a faster and voltage-independent component ($\tau = 37$ ms). The linear relationship between the τ^{-1} of the current decay and the cilnidipine concentration provided a value of 471 nM for the dissociation constant in the open state, suggesting that the current decay is mediated by one-to-one lower affinity binding of cilnidipine molecules to their binding site. The present study demonstrates that structurally related DHPs act in distinct ways to inhibit the L-type channel in the resting, open and inactivated states. Cilnidipine and some related DHPs probably exert their blocking action on the open channel by binding to a receptor distinct from the known DHP-binding site.

Keywords: Dihydropyridine, Ca^{2+} channel, Ca^{2+} antagonist, Open channel, Channel blocker

Amino acids important for dihydropyridine (DHP) binding to voltage-gated L-type Ca^{2+} channels have been identified by means of photoaffinity labelling, a chimerical approach and site-directed mutagenesis (1, 2). Detailed studies from several laboratories have demonstrated that these amino acids are located in the fifth segment of the third repeat (IIIS5), in IIIS6 and in IV6, all within the α_1 -subunit of the L-type Ca^{2+} channel (3–6). The mechanism by which DHPs interact with Ca^{2+} channels has been interpreted as showing state-dependent binding of these compounds to their receptors, since they have different affinities for the resting, open and inactivated states (7–9). It is well documented that DHPs act preferentially on the inactivated state with high-affinity binding, which is favored at depolarized membrane potentials (10, 11). On the other hand, heterogeneous types of inhibition by Ca^{2+} blockers have also been reported in previous studies, which produced evidence of DHP-induced acceleration of current decay during step pulses when relatively large doses of DHPs were used (12–14). An open-channel blockade (15) or an acceleration of

current inactivation (12) has been proposed as the mechanism underlying this effect on current decay.

The importance of the exact DHP structure for state-dependent inhibition has not been investigated to date. Cilnidipine is a long-lasting antihypertensive compound (16, 17) that blocks L- and N-type Ca^{2+} channels in dorsal root ganglion neurons (18). This molecule possesses a core structure with a unique, bulky moiety (3-phenyl-2-propene-1-yl-oxycarbonyl) bound to the C5 position of the DHP ring. Nimodipine, which shares the same core structure, has a relatively small substitution at the C5 position (isopropyl-oxycarbonyl) (Fig. 1). With the original backbone structure retained, a structural difference of this type might alter the manner in which inhibition of Ca^{2+} channels is produced. A fascinating example is that cilnidipine, but not nimodipine, is capable of inhibiting a different class (α_{1B}) of Ca^{2+} channel (18–20), suggesting high complementarity between these compounds and the binding site (receptor). Heterogeneous binding sites for DHPs are expected for this channel, as for the L-type Ca^{2+} channel. A recent study has shown that isradipine causes current decay in a triple mutant of the α_1 -subunit of the L-type Ca^{2+} channel that lacks a high-affinity binding site for DHPs (21). Those au-

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Fig. 1. Chemical structures of cilnidipine, D-342, nimodipine and D-69. All compounds employed in the present study were racemic forms.

thors supposed the existence of a low-affinity binding site for DHPs mediating a different type of action by these drugs. Thus, it is plausible that different DHP derivatives may modify channel activity in different ways, depending on whether they can or cannot recognize the structural difference between the two types of binding sites.

In the present report, we investigated state-dependent inhibition of L-type Ca^{2+} channels by structurally related compounds using A7r5 cells, an established cell-line derived from the smooth muscle of rat thoracic aorta, and employing a conventional patch-clamp technique. Our aims were as follows: i) to examine whether or not heterogeneous types of inhibition by cilnidipine can be obtained depending on the channel state; ii) if so, to compare this molecule with 3 structurally related compounds (nimodipine, D-342 and D-69) of which the D-342 and D-69 are cilnidipine derivatives with an additional C=C bond instead of the phenyl ring in the cilnidipine-specific side-chain and a branched methyl group bound on the side-chain, respectively; iii) to distinguish the various types of channel inhibition by examining the concentration-dependency of the steady-state blockade by analyzing the kinetics of the current decay and by estimation of the binding affinities between the channel and the drugs in the resting, open and inactivated states.

MATERIALS AND METHODS

Cell culture

The A7r5 cell line, derived from rat thoracic aorta, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (Dainippon Pharm. Co., Osaka) in the presence of 10% heat-inactivated (56°C, 40 min) fetal calf serum (ICN Biomedicals, Inc., Aurora, OH,

USA), 1.7 mM L-glutamine, 50 $\mu\text{g} \cdot \text{ml}^{-1}$ gentamicin sulphate (Gibco, Grand Island, NY, USA) and non-essential amino acids in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Cells were plated onto poly-L-lysine-coated glass coverslips (Matsunami, Osaka) placed in a tissue-culture dish (Becton Dickinson, Franklin Lakes, NJ, USA) at a density of 2.0×10^4 cells per dish. The cells were cultured for more than 1 week before use.

Electrophysiology

The A7r5 cells were transferred to a recording chamber mounted on the stage of a microscope (DM IRB; Leitz, Wetzlar, Germany). The chamber, which had a volume of 1 ml, was continuously perfused with bathing solution at a rate of 2.5 $\text{ml} \cdot \text{min}^{-1}$. Currents were recorded via an EPC-9 amplifier (HEKA Elektronik GmbH, Lambrecht, Germany) in a whole-cell configuration using a conventional patch-clamp technique (22). Electrode pipettes were made from borosilicate glass capillaries (1.5 mm OD; Narishige Scientific Instrument Lab., Tokyo) using a Flaming-Brown micropipette puller (model P-87; Sutter Instruments, Novato, CA, USA) and then heat-polished (MF-83, Narishige). Their resistance was 2 to 4 M Ω in the bathing solution when filled with the pipette solution described below. They were handled with the aid of an electrically driven micromanipulator (Steuerpult 5171; Eppendorf, Hamburg, Germany). Currents were filtered at 5 kHz and digitized online at 20 kHz using a Power Macintosh G3 DT266 (Apple Computer, Inc., Cupertino, CA, USA). All experiments were conducted at room temperature (22–25°C).

Solutions

To measure Ba^{2+} currents during whole-cell recording, A7r5 cells were perfused with a bathing solution containing: 162 mM tetraethylammonium chloride (TEA-Cl), 10 mM

BaCl₂, 10 mM glucose and 10 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES). Patch pipettes were filled with a solution containing: 125 mM CsOH, 125 mM L-aspartic acid, 5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), 10 mM HEPES, 5 mM ATP · 2Na salt and 5 mM MgCl₂. The pH of the bathing solution was adjusted to 7.4 using TEA-OH and that of the pipette solution to 7.2 using CsOH.

Drugs

The chemical structures of cilnidipine, the two cilnidipine derivatives (D-69, D-342) and nimodipine are shown in Fig. 1. Cilnidipine, D-69 and D-342 (synthesised by Fuji-Rebio Inc., Tokyo), nifedipine (Sigma Chemical Co., St. Louis, MO, USA) and nimodipine (Calbiochem, San Diego, CA, USA) were dissolved in DMSO (Sigma Chemical Co.) as 10 mM stock solutions, which were stored at -20°C. The stock solutions were diluted with the bathing solution to make the desired final concentration. The final concentration of DMSO was less than 0.1%, which did not affect the amplitude or kinetics of the Ba²⁺ current recorded in A7r5 cells. These compounds were applied by superfusion to the bath. Due to the light-sensitivity of the drugs, all were prepared and stored in the dark, and the perfusion lines containing these drugs were light-shielded.

Data analyses

Capacitative and leak currents were eliminated by using the P/4 method (23). The open-channel blocking action of the drugs was assessed by calculating the difference between the current amplitudes at peak and at the end of the pulse. All of the data entered into the analysis represented the maximum effects reached after application of the relevant drug. Data were analyzed using the PulseFit program (HEKA Elektronik). Curve fitting was carried out using the programs Igor Pro (WaveMetrics, Inc., Lake Oswego, OR, USA) and Kaleida Graph (Synergy Software, Reading, PA, USA) programs. Dose-response curves were fitted by the Hill equation:

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

where IC₅₀ is the concentration at half-blockade, [D] is the drug concentration and n_H is the Hill coefficient.

The current traces evoked by depolarizing pulses (I(t)) were fitted using a single or double exponential function:

$$I(t) = A * \exp(-t/\tau) + C$$

or

$$I(t) = A_1 * \exp(-t/\tau_1) + A_2 * \exp(-t/\tau_2) + C,$$

where t is the time from the onset of the pulse, A and τ are the amplitude and time constant of each exponential component, and C is the amplitude of the non-decaying component.

Steady-state inactivation curves were fitted by the Boltzmann equation. The relative amplitude of the L-type current

(I) observed at one of a number of voltages (V) of prepulse was expressed as:

$$I / I_{\max} = (I_{\max} - C) / (1 + \exp((V - V_{\text{half}})/k)) + C,$$

where I_{max} is the current amplitude without a prepulse; V_{half}, the prepulse potential that halved the amplitude of the inactivating component of the L-type current; k, the slope factor; and C, the fraction of the current represented by the non-inactivating component.

Kinetics analysis was performed on the time course of the current decay induced by cilnidipine. After double-exponential fitting, the faster τ values were plotted against the cilnidipine concentration. If one molecule of the drug (D) binds to one binding site of receptor molecule (R), then the second-order reaction (D + R ↔ DR) would apply. Therefore, the reaction rate needed to produce the channel-drug complex is described as follows:

$$d[\text{DR}] / dt = k_{+1}[\text{D}][\text{R}] - k_{-1}[\text{DR}],$$

where k₊₁ and k₋₁ are the association and dissociation rate constants, respectively. The dissociation constant (K_d) can be estimated from these two rate constants: K_d = k₋₁ / k₊₁. If the above assumption holds true, the time constant is expressed as follows: τ = 1 / k₊₁ (K_d + [D]). Therefore, τ⁻¹ should be a linear function of [D], described by: τ⁻¹ = k₋₁ + k₊₁ [D]. In the present study, the reciprocal of τ (τ⁻¹) was plotted as a function of the drug concentration. The slope and y-intercept are, then, taken as k₊₁ and k₋₁, respectively, if the regression function is linear.

Statistics

All values are presented as means ± S.E.M. (n, number of observations). Statistical analysis was performed using a paired or unpaired *t*-test when 2 groups were compared or by one-way analysis of variance (ANOVA) followed by a *post hoc* Bonferroni's *t*-test for more than 3 groups. A *P* value less than 0.05 was considered to be statistically significant.

RESULTS

A7r5 cells have been reported to express two distinct types of Ca²⁺ channels (L- and T-types) (24). In preliminary experiments, we examined the current-voltage relationship of Ca²⁺ channels in A7r5 cells and found that there were two different inward currents (low- and high-voltage threshold currents). Application of 10 μM cilnidipine or nifedipine to the cells, which exhibited the low-voltage threshold current, completely inhibited the high-voltage threshold current. However, no effect was observed on the low-voltage threshold current by these DHPs. On the other hand, the inward current in the cells which solely exhibited the high-voltage threshold current was completely inhibited by these DHPs. Including these preliminary results, only 3 out of 201 cells exhibited the low-voltage threshold; thus,

in the present paper, we performed the experiments using the other 198 cells.

Effects of cilnidipine and nimodipine on the L-type current

To examine the effect of cilnidipine and nimodipine on the L-type current, a Ba^{2+} current was evoked by depolarizing pulses to 10 mV from a holding potential of -80 mV (50-ms duration). Figure 2A shows raw current traces and time courses obtained in the absence and presence of cilnidipine. Superfusion with 10 nM and 1 μM cilnidipine reduced the peak amplitude of the Ba^{2+} current in a concentration-dependent manner. With a low concentration of cilnidipine (10 nM), the reduction in the amplitude of the inward current measured at peak (17%) was of the same magnitude as that measured at the end of the pulse (19%). It was noteworthy, however, that 1 μM cilnidipine had a more profound inhibitory effect on the current amplitude measured at the end of the pulse (73%) than on that measured at the peak (51%), as demonstrated in a typical trace (c) (Fig. 2A) that shows a faster current decay than trace (a). On the other hand, the inhibitory effect of 100 nM nimodipine on the amplitude of the Ba^{2+} current was much the same whether it was measured at the peak (50%) or at

the end of the pulse (59%) (Fig. 2B). Thus, although this concentration of nimodipine produced more than 50% inhibition, it caused no detectable change in the shape of the Ba^{2+} current, in contrast to cilnidipine. A subsequent application of 10 μM nifedipine further inhibited the Ba^{2+} current so that only a percentage of the initial current remained; this was thereafter completely inhibited by superfusion with 100 μM CdCl_2 (Fig. 2: A and B). These results suggest that most of the current in the present preparation was of the L-type.

Current-voltage relationships were obtained in the absence and presence of 1 μM cilnidipine (Fig. 3). In the control, the current-voltage relationship was almost the same at the end of the pulse as at the peak. Cilnidipine inhibited the current amplitude more profoundly at the end of the pulse than at the peak over the entire range of effective potentials (-30 to 40 mV).

Relationship between the amplitude of the L-type current and concentration of four DHP derivatives

We next examined the concentration-dependent effects of the four DHP derivatives on the L-type current. Figure 4 shows the relationship between the amplitude of the L-type

Fig. 2. Effects of cilnidipine (A) and nimodipine (B) on the L-type current. Ba^{2+} current was evoked by depolarizing pulses to 10 mV from a holding potential of -80 mV (50-ms duration). Time course of change in the amplitude of Ba^{2+} current is shown for various concentrations of cilnidipine and nimodipine; current was measured at peak (open circles) and at the end of the pulse (closed circles). To permit assessment of the total DHP-sensitive and Ca^{2+} -dependent components of the current, 10 μM nifedipine and 100 μM CdCl_2 , respectively, were also applied to the cell. Upper panels in A and B show typical current traces obtained at the times indicated by the dotted lines in the graphs.

Fig. 3. Current-voltage relationships obtained in the absence (circles) and presence (triangles) of $1 \mu\text{M}$ cilnidipine. Plots show current amplitudes measured at peak (open symbols) and at the end of the pulse (filled symbols), each as the average of 12 observations. Voltage protocol is indicated in the inset. Current amplitude is expressed relative to maximal value evoked by the depolarizing pulse in control.

current and drug concentration for cilnidipine (A), D-69 (B), D-342 (C) and nimodipine (D). Each drug reduced

the amplitude of the Ba^{2+} current at peak and at the end of the pulse in a concentration-dependent manner (10 nM – $10 \mu\text{M}$). At higher concentrations (1 and $10 \mu\text{M}$), cilnidipine and D-342 significantly inhibited the current amplitude measured at the end of pulse to a greater extent than that measured at the peak. Estimated IC_{50} values and Hill coefficients for the four DHP derivatives are shown in Table 1. The rank order of the IC_{50} values for the effects on the peak and end-of-pulse amplitudes was: for I_{Peak} , D-69 = nimodipine > D-342 > cilnidipine; for I_{End} , D-69 = cilnidipine > D-342 > nimodipine. The presence of a Hill coefficient less than unity in the cilnidipine-, D-342- and D-69-treated groups suggests that multiple binding sites may be involved in the inhibitory mechanism.

Steady-state inactivation curves for the L-type current

Next, the effects of cilnidipine and nimodipine were examined on the availability of L-type channels. A long prepulse (3 s) of various potentials was applied every 15 s just before the test pulse (to 10 mV , 50 ms in duration). As shown in Fig. 5, the amplitude of the Ba^{2+} current evoked by the test pulse decreased as the amplitude of the prepulse was increased. Perfusion with $1 \mu\text{M}$ cilnidipine and 100 nM

Fig. 4. Relationships between amplitude of Ba^{2+} current and drug concentration: cilnidipine (A), D-69 (B), D-342 (C) and nimodipine (D). Ba^{2+} current was evoked by depolarizing pulses to 10 mV from a holding potential of -80 mV (50-ms duration). To obtain the DHP-sensitive component (L-type current), $10 \mu\text{M}$ nifedipine and $100 \mu\text{M}$ CdCl_2 were successively applied and the net amplitude of the L-type current was estimated by subtracting the Ba^{2+} current recorded in $10 \mu\text{M}$ nifedipine from that in control or with a test drug. Ba^{2+} -current inhibition (%) is expressed in a relative manner, the net amplitude obtained in the presence of drug being divided by that obtained in the control. The line was fitted by the Hill equation (see Materials and Methods) using the least-squares method. In case of Fig. 4D, an infinitely small concentration of nimodipine was assumed to produce no current inhibition. Each point represents mean $\pm$ S.E.M. for 3–8 observations (cilnidipine), 3–7 observations (D-69), 3–5 observations (D-342) or 2–5 observations (nimodipine). * $P < 0.05$, ** $P < 0.01$ vs peak amplitude.

Table 1. IC₅₀ and Hill coefficient for four DHP derivatives

		Cilnidipine	D-342	D-69	Nimodipine
Peak	IC ₅₀ (nM)	190	132	47	96
	Hill coefficient	0.324	0.539	0.671	0.919
End	IC ₅₀ (nM)	66	82	61	100
	Hill coefficient	0.512	0.715	0.733	0.968

Values were calculated from the data shown in Fig. 4.

nimodipine, which produced approximately 60% and 50% inhibition of the peak Ba²⁺ current, respectively (Fig. 4), markedly shifted the steady-state inactivation curve to the left. The curves obtained in the absence and presence of either cilnidipine or nimodipine provided the following V_{half} values: control, -22.1 ± 1.8 mV; cilnidipine, -47.9 ± 0.5 mV; nimodipine, -47.8 ± 0.5 mV. There was no significant difference between the shifts in V_{half} produced by cilnidipine and nimodipine at these concentrations (*P* > 0.05), and the *k* values were not different among the three conditions (*P* > 0.05).

Kinetic analysis of the current decay seen in the presence of cilnidipine

To investigate the actions of cilnidipine in more detail, especially its effect on the decaying component, we used two different protocols. First, current decay was quantified

Fig. 5. Steady-state inactivation curves for L-type current obtained in the absence or presence of drug (cilnidipine or nimodipine). The voltage protocol is indicated in the inset. Relative amplitude of the Ba²⁺ current evoked by a test pulse (to 10 mV, 50-ms duration) is plotted against the amplitude of the pre-pulse potential (to various potentials, 3-s duration). The current amplitude (ordinate) is expressed in a relative manner, the amplitude of the current evoked without a prepulse being normalized as 1.0. Each symbol indicates the mean ± S.E.M. (control, *n* = 9; cilnidipine, *n* = 6; nimodipine, *n* = 6). The theoretical curves were drawn using the Boltzman equation (see Materials and Methods). The estimated parameters are as follows: control: V_{half} = -22.1 ± 1.8 mV, *k* = -14.5 ± 1.0 mV; cilnidipine: V_{half} = -47.9 ± 0.5 mV, *k* = -9.1 ± 0.5 mV; nimodipine: V_{half} = -47.8 ± 0.5 mV, *k* = -8.8 ± 0.4 mV. **P* < 0.05, ***P* < 0.01, vs the control value at the same potential.

Fig. 6. Analysis of decaying component observed in the presence of cilnidipine. Ba²⁺ current was evoked by depolarizing pulses to 10 mV from a holding potential of -80 mV (50-ms duration). A: relationship between decaying component and cilnidipine concentration. The amplitude of the decaying component in the absence or presence of cilnidipine (F_{Cont} or F_{Cil}) was quantified by subtracting the amplitude measured at the end of the pulse (I_{End}) from the peak current (I_{Peak}). Then, the ratio of F values (F_{Cil}/F_{Cont}) was plotted against the cilnidipine concentration. The curve was fitted with a Hill equation using a least-squares method. Each point represents a mean ± S.E.M., the number of observations being 3 (for 0.01 μM), 3 (for 0.1 μM), 5 (for 1 μM) or 8 (for 10 μM). B: scatter graph showing the relationship between the reciprocal of the time constant (τ⁻¹) and cilnidipine concentration. After curve-fitting for the entire Ba²⁺ current by means of a double exponential function (see Materials and Methods), the faster decaying component was used for the graph. The values obtained for the faster component indicate acceleration of current decay by cilnidipine. The mean values at the various cilnidipine concentrations are fitted by a linear function with a correlation coefficient of 0.90 (*y* = 24.9*x* + 11.7).

by calculating the peak amplitude of the Ba^{2+} current (expressed as F), which decreased during the 50-ms pulse. Then, the ratio of the F values ($F_{\text{Cil}}/F_{\text{Cont}}$) obtained in the absence and presence of cilnidipine was plotted against the cilnidipine concentration. Current was evoked by depolarizing pulses to 10 mV from a holding potential of -80 mV. Figure 6A clearly demonstrates that the $F_{\text{Cil}}/F_{\text{Cont}}$ values increased in a concentration-dependent manner, indicating the presence of a cilnidipine-sensitive decay in the Ba^{2+} current. The curve obtained from 19 cells was fitted by a Hill equation using a least-squares method, giving the following values: $\text{IC}_{50} = 220$ nM and Hill coefficient = 0.93. The IC_{50} value calculated for D-342 was 50 nM. On the other hand, no clear reduction in F value ratio was obtained with D-69 or nimodipine over the concentration ranges used in the present experiments.

Secondly, we quantified the current decay by calculating its time constant. Figure 6B shows the relationship between the reciprocal of the time constant (τ^{-1}) of the fast current decay and the cilnidipine concentration. Smaller τ values are likely to indicate drug-receptor interaction mediating the open-channel blockade, as shown in the next paragraph. The data representing τ^{-1} as a function of cilnidipine concentration were well scattered around a straight line, with the best fit being to the following linear function: $y = 24.921x + 11.743$ ($r = 0.90$). The values obtained for the slope factor and y-intercept represent the association and dissociation rate constants ($11.7 \text{ ms}^{-1} \cdot \mu\text{M}^{-1}$ and 24.9 ms^{-1} , respectively). Thus, the K_d value was estimated to be 471 nM, which is close to the IC_{50} obtained in Fig. 6A.

Effect of cilnidipine and nimodipine on open-channel-block and inactivation kinetics of the L-type current

Figure 7 shows current traces obtained in the absence and presence of cilnidipine or nimodipine using 3 different amplitudes of voltage step (1-s duration). The current traces evoked by various amplitudes of depolarizing pulse in the absence of drug (control) were well fitted by a single-exponential function. On the other hand, the current traces obtained in the presence of cilnidipine did not fit such a function, but did fit a double-exponential function. The appearance of a faster decaying component (τ_{Cil}) suggests a cilnidipine-specific action on the time course of the current decay. Current traces obtained in the presence of nimodipine were well fitted by a single-exponential function, indicating that nimodipine did not have the novel action observed for cilnidipine.

Table 2 summarizes the effects of cilnidipine, D-69 and nimodipine on the time constant of the decay observed in the L-type current (evoked by a depolarizing pulse to 10 mV). The τ_{Cil} value, observed only in the presence of cilnidipine, was smaller than the τ values obtained for the other drugs (140–200 ms). There was no significant difference among

Fig. 7. Effects of $1 \mu\text{M}$ cilnidipine and 100 nM nimodipine on inactivation kinetics of the L-type current. A: current traces obtained in the absence (C) and presence of cilnidipine (Cil) or nimodipine (Nim). Superimposed Ba^{2+} current was evoked by depolarizing pulses to 3 different potentials (-20 , 0 and 20 mV) for 1-s duration from a holding potential of -80 mV. The currents were fitted with single (control and nimodipine) or double exponential functions (cilnidipine). (see “data analyses” in “Materials and Methods”). A, A_1 , A_2 , C indicate fraction of current amplitude when the maximum amplitude of each trace is registered as 1.0. Values of each parameter were as follows: control (-20 mV) $A = 0.31$, $\tau = 386$ ms, $C = 0.69$, cilnidipine (-20 mV) $A_1 = 0.11$, $\tau = 428$ ms, $A_2 = 0.76$, $\tau_{\text{Cil}} = 22$ ms, $C = 0.13$; control (0 mV) $A = 0.83$, $\tau = 145$ ms, $C = 0.17$, cilnidipine (0 mV) $A_1 = 0.38$, $\tau = 149$ ms, $A_2 = 0.53$, $\tau_{\text{Cil}} = 34$ ms, $C = 0.09$; control ($+20$ mV) $A = 0.80$, $\tau = 138$ ms, $C = 0.20$, cilnidipine ($+20$ mV) $A_1 = 0.61$, $\tau = 95$ ms, $A_2 = 0.25$, $\tau_{\text{Cil}} = 30$ ms, $C = 0.14$; control (-20 mV) $A = 0.55$, $\tau = 334$ ms, $C = 0.45$, nimodipine (-20 mV) $A = 0.84$, $\tau = 368$ ms, $C = 0.16$; control (0 mV) $A = 0.81$, $\tau = 196$ ms, $C = 0.19$, nimodipine (0 mV) $A = 0.96$, $\tau = 165$ ms, $C = 0.04$; control ($+20$ mV) $A = 0.76$, $\tau = 177$ ms, $C = 0.24$, nimodipine ($+20$ mV) $A = 0.98$, $\tau = 151$ ms, $C = 0.02$. B: relationships between the time constant of current decay and the voltage of the depolarizing pulse in the absence and presence of cilnidipine or nimodipine. Each time-constant value was obtained using the protocol described for panel A. τ_{Cil} indicates the smaller time constant that appeared only in the presence of cilnidipine. Each point represents the mean $\pm$ S.E.M. for 9–17 observations (control), 9–11 observations (cilnidipine) or 4–8 observations (nimodipine). * $P < 0.05$, ** $P < 0.01$, control vs cilnidipine or nimodipine at -20 mV.

Table 2. Effects of cilnidipine, nimodipine and D-69 on decay of L-type current

Blocker	n	τ_{Cil} (ms)	Fraction (%)	τ (ms)	Fraction (%)	Residual Fraction (%)
Control	78			194.96 $\pm$ 6.82	83.96 $\pm$ 1.39	16.04 $\pm$ 1.39
Cilnidipine 1 μM	34	36.69 $\pm$ 2.21	63.06 $\pm$ 3.20	201.50 $\pm$ 23.04	30.36 $\pm$ 2.20	6.58 $\pm$ 1.82
Nimodipine 100 nM	16			141.04 $\pm$ 7.92	92.60 $\pm$ 1.23	7.40 $\pm$ 1.23
D-69 100 nM	12			159.30 $\pm$ 25.80	93.05 $\pm$ 5.04	6.95 $\pm$ 5.04

Ba²⁺ current was evoked by a 1-s depolarizing pulse to 10 mV from a holding potential of -80 mV.

the τ values observed in the absence or presence of DHPs ($P > 0.05$). The fraction of the amplitude of the total current represented by the τ component was nearly the same in control, in the presence of nimodipine and in the presence of D-69 (around 80% to 90%). The residual fraction, implying a non-inactivated or extreme-slowly inactivated component was 6–16% for all four conditions obtained. In case of cilnidipine, the τ_{Cil} component represented 63% of the total current, and the sum of the fractional values for the τ_{Cil} and τ components (93%) was about the same as the fractional value of the τ component in the other conditions.

As shown in Fig. 7B, the τ values obtained in the control and in the presence of nimodipine were dependent on membrane voltage. At -20 mV, the τ values in the control (open circles) and in nimodipine-treated cells (open diamonds) were 370 and 300 ms, respectively, while they were within the range 150 to 230 ms at positive voltages. The time constant of the slower-decaying component obtained in the presence of cilnidipine (open triangle) also decreased as the amplitude of the depolarization increased. At -20 mV, the τ values obtained in the presence of cilnidipine ($P < 0.01$) and nimodipine ($P < 0.05$) were significantly smaller than that obtained in the control. It was noteworthy that τ_{Cil} , the time constant of the faster decaying component, was almost constant over the range of potentials tested (-20 to +30 mV). These results suggest that cilnidipine has a specific action on the decaying kinetics of the L-type channel in addition to the characteristic inhibitory action observed with other DHPs.

DISCUSSION

In A7r5 cells, two types of voltage-activated Ca²⁺ channels, T- and L-types, are expressed, and they are distinguished by several properties such as the decay time of the current or the sensitivity to DHP-type Ca²⁺-antagonists (24). The present experiments also found two types of channels. The channels that showed very fast decay in time course and insensitive to nifedipine were detected in only 3 of the tested cells. Therefore, the experiments were carried out mainly on the effects of cilnidipine on L-type Ca²⁺ channels.

The present study demonstrates two distinct ways in which cilnidipine-related compounds can block voltage-dependent L-type Ca²⁺ channels. One of the actions involves inhibition of the peak current (I_{Peak}) and the other, a time-dependent inhibition of the current, i.e., a fast decay in the current seen during a voltage step. Our results reveal that these two types of blockade differ in concentration-dependency, structure-specificity and voltage-dependency. The most interesting finding in the present report is that the DHP binding to L-type Ca²⁺ channels that induces enhanced current decay requires a specific compound structure. Recent papers have shown that an enantioselectivity of nifedipine and isradipine is involved in the blockade of I_{Peak} (25, 26). A structural selectivity of drug action is generally considered to reflect the structure of the binding site and the interaction between the drug and the binding site. While cilnidipine clearly induced a fast current decay, the structurally-related compounds nimodipine and D-69 failed to alter the kinetics of the Ca²⁺ current. The rank order of potencies for actions on current decay (roughly estimated from $F_{\text{drug}}/F_{\text{cont}}$ value) was D-342 > cilnidipine $\gg$ D-69 = nimodipine. Such a structural selectivity implies the existence of a specific binding site mediating this effect on current decay. In contrast, the rank order of IC_{50} values for the inhibition of I_{Peak} was D-69 = nimodipine > D-342 > cilnidipine. A plausible explanation for these different rank orders is that two different types of binding sites may be involved in the mechanisms underlying the two types of response.

The present study clearly shows that the fast current decay induced by cilnidipine can be interpreted as reflecting a simple receptor-drug interaction. Previously, Roulet et al. (27) conducted kinetic analyses to obtain association and dissociation rate constants for the open-channel blocking effect of farnesol on the N-type Ca²⁺ channel. We adopted their methodology. Assuming that the drug binds to the receptor with a stoichiometry of 1:1, the function between the reciprocal of the time constant and the concentrations of drug obtained from kinetic analysis should be fitted with a linear regression line. The experimentally obtained relationship (for cilnidipine) was, indeed, described by a linear regression line (Fig. 6B), consistent with the assumption

that one molecule of drug binds to a receptor within the channel and thus influences the kinetics of channel opening. By using this function, association (k_{+1}) and dissociation (k_{-1}) rate constants for the interaction between the drug and the receptor were calculated and these yielded a K_d value of 471 nM (termed K_O , Fig. 8). This value is in good agreement with the IC_{50} value obtained from the equilibrium dose-response relationship for the decaying component of the current (Fig. 6A and Table 1).

An acceleration of current decay has previously been documented in many reports (9, 12, 14, 15, 21). On the assumption of state-dependent binding of DHPs, most papers ascribed such effects to an open-channel block. Here, we propose that the observed cilnidipine-induced current decay is also attributable to an open-channel block for the following reasons. First of all, the K_d values for cilnidipine in the resting state (K_R) and the inactivated state (K_I) were not the same as the K_d for the action of cilnidipine on the fast current decay (K_O). Inhibition of I_{peak} by cilnidipine is considered mainly to reflect the effect of this compound on the channel in the resting state. This is because the holding potential was at a negative voltage (-80 mV) and the frequency of the depolarizing steps (0.067 Hz) was low enough to allow the channel to switch from the open or inactivated state to the resting state (Figs. 3 and 5) (8, 21). The K_R value was thus estimated to be 190 nM after fitting the dose-response curve to a Hill function (Table 1). Together with the K_R value, the shift in the steady-state inactivation curve produced by cilnidipine provides a K_I value of 12 nM (according to the method of estimation previously used by Bean et al. (28)). Similarly, we obtained K_R and K_I values for nimodipine of 96 and 4 nM, respectively. The high affinities for the inactivated state obtained for cilnidipine and nimodipine in the present experiment are in a good agreement with the results in the literature (10, 11). Figure 8 depicts the simplest model in which three

Fig. 8. Simplified model for state-dependent inhibition of L-type Ca^{2+} channel by cilnidipine (Cil) and nimodipine (Nim). The values of the apparent dissociation constants are $K_R = 190$ nM, $K_O = 471$ nM, $K_I = 12$ nM (cilnidipine) and $K_R = 96$ nM, $K_I = 4$ nM (nimodipine). R, resting; O, open; I, inactivated state. Asterisks indicate the channel states to which drug binds.

states of the Ca^{2+} channel are bound by cilnidipine. K_R , K_O and K_I are considered to represent different affinities for the transition of the channel from drug-unbound (R, O and I) to drug-bound (R^* , O^* and I^*) states. The estimated K_O value (471 nM) obtained for cilnidipine in this study (Fig. 6B) was 2.5- and 39-fold greater than the K_R and K_I , respectively. Accordingly, the cilnidipine-induced current decay cannot be interpreted as indicating binding to either the resting or inactivated state.

Comparing the decaying kinetics in the presence or absence of a DHP is a powerful tool in attempts to characterize the time-dependent interaction between a drug and the open/inactivated states of the channel. The time courses obtained in control and in the presence of nimodipine or D-69 were described simply as the summation of monoexponential (τ) and residual components (Fig. 7). The striking difference between the cilnidipine-induced effect and the others was that the time courses were adequately fitted by the summation of biexponential (τ_{Cil} and τ) and residual components. The rapidly decaying component (τ_{Cil}) is likely to be a specific action of cilnidipine since τ_{Cil} was newly created under this compound and failed to exhibit voltage-dependency. This phenomenon is consistent with the previous finding that isradipine behaves as an open-channel blocker and has an effect that is not voltage-dependent (21). After all these results have been taken into a consideration, an action on the open channel is the most probable interpretation.

The τ values, which were similar in all conditions tested ($140 - 200$ ms at 10 mV), appear to represent the time course of voltage-dependent inactivation of the channel since they were larger at more negative potentials (Fig. 7B) (10, 29). The presence of a residual component during 1-s steps after double-exponential fitting would imply extremely slow inactivation of the channel with a larger time constant (10). The ability of cilnidipine and nimodipine to affect the inactivation kinetics is likely to represent a common characteristic of DHP-type Ca^{2+} blockers. These results support the previous claims that DHPs favour the inactivation state of L-type Ca^{2+} channels (10, 11). The finding that a shift in the steady-state inactivation curve was produced by each of these two compounds confirms such a state-selective action of most DHPs. Although Ca^{2+} -dependent inactivation is also expected (10, 29), this type of inactivation can be mostly neglected under the present experimental conditions. This is because Ba^{2+} was used in the bath solution as a charge carrier and because BAPTA was included in the pipette solution to chelate intracellular Ca^{2+} . The relationship between τ values and membrane voltage, in fact, did not exhibit the U-shaped curve that would have indicated that inactivation is retarded by a weakened Ca^{2+} drive at potentials toward its reversal potential (30).

The structurally related compounds D-69 and nimo-

dipine apparently could not elicit the fast current decay, while D-342 did so to a lesser extent than cilnidipine. It is of interest that the presence of a bulky moiety in the side chain does not necessarily confer on the ability of DHPs to produce a fast decay (Fig. 1). It is noteworthy that both of the less potent compounds possess a methyl group next to a carboxylate moiety. This raises the possibility that these methyl groups may interfere with the drug's binding to the specific site (although admittedly a very limited number of compounds was tested in the present study). Because of such a possible steric hindrance, a much higher concentration of D-69 and nimodipine may be required to induce the fast decay than that required to inhibit I_{Peak} . In the most extreme case, both compounds may entirely lack the ability to induce a fast decay. We cannot absolutely deny the alternative possibility that an action of D-69 and nimodipine producing a fast decay of about the same magnitude as that produced by cilnidipine may have been masked by a more potent effect of these drugs on I_{Peak} . In order to examine which possibility is the more likely, it would be best to utilize mutant L-type channels in which the high-affinity binding sites for DHPs are missing due to replacement of several amino acids in the IV6 domain (26). This procedure should enable us to identify the domain that is essential for cilnidipine binding to elicit current decay in the L-type Ca^{2+} channel.

DHP compounds with the unique cilnidipine-like profile are likely to cause a progressive reduction in Ca^{2+} influx during depolarization, in addition to a tonic block of the Ca^{2+} channel in the resting condition. This leads us to speculate that a cilnidipine-like compound that is effective in the submicromolar range, might shorten the action potential duration by an immediate block of the open channel when the drug was applied to the excitable cells. Since use-dependent blockers inhibiting Ca^{2+} channels are known to act preferentially during high electrical activity (e.g., cardiac arrhythmia), intrinsic gating profiles that affect the state of the channel may play a key role in determining the tissue-specificity of drug sensitivity (13).

In conclusion, the present study has demonstrated that structurally related DHPs produce distinct types of inhibition of the L-type Ca^{2+} channel in the resting, open and inactivated states. Cilnidipine and some related drugs are likely to exert their blocking action on the open channel (leading to faster current decay) by structure-dependent binding to a different receptor with an affinity lower than that of the previously identified DHP-binding site.

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REFERENCES

- 1 Nakayama H, Taki M, Striessnig J, Glossmann H, Catterall WA and Kanaoka Y: Identification of 1,4-dihydropyridine binding regions within the α_1 subunit of skeletal muscle Ca^{2+} channels by photoaffinity labeling with diazepam. *Proc Natl Acad Sci USA* **88**, 9203 – 9207 (1991)
- 2 Striessnig J, Grabner M, Mitterdorfer J, Hering S, Sinnegger MJ and Glossmann H: Structural basis of drug binding to L Ca^{2+} channels. *Trends Pharmacol Sci* **19**, 108 – 115 (1998)
- 3 Schuster A, Lacinova L, Klugbauer N, Ito H, Birnbaumer L and Hofmann F: The IVS6 segment of the L-type calcium channel is critical for the action of dihydropyridines and phenylalkylamines. *EMBO J* **15**, 2365 – 2370 (1996)
- 4 Bodi I, Yamaguchi H, Hara M, He M, Schwartz A and Varadi G: Molecular studies on the voltage dependence of dihydropyridine action on L-type Ca^{2+} channels. *J Biol Chem* **272**, 24952 – 24960 (1997)
- 5 Ito H, Klugbauer N and Hofmann F: Transfer of the high affinity dihydropyridine sensitivity from L-type to non-L-type calcium channel. *Mol Pharmacol* **52**, 735 – 740 (1997)
- 6 Peterson BZ, Johnson BD, Hockerman GH, Acheson M, Scheuer T and Catterall WA: Analysis of the dihydropyridine receptor site of L-type calcium channels by alanine-scanning mutagenesis. *J Biol Chem* **272**, 18752 – 18758 (1997)
- 7 McDonald TF, Pelzer D and Trautwein W: Cat ventricular muscle treated with D600: characteristics of calcium channel block and unblock. *J Physiol (Lond)* **352**, 217 – 241 (1984)
- 8 Uehara A and Hume JR: Interactions of organic calcium channel antagonists with calcium channels in single frog atrial cells. *J Gen Physiol* **85**, 621 – 647 (1985)
- 9 Hering S, Beech DJ, Bolton TB and Lim SP: Action of nifedipine or BAY K8644 is dependent on calcium channel state in single smooth muscle cells from rabbit ear artery. *Eur J Physiol* **411**, 590 – 592 (1988)
- 10 Kass RS and Sanguinetti MC: Inactivation of calcium channel current in the calf cardiac purkinje fiber. *J Gen Physiol* **84**, 705 – 726 (1984)
- 11 Bean BP: Nitrendipine block of cardiac calcium channels: high-affinity binding to the inactivated state. *Proc Natl Acad Sci USA* **81**, 6388 – 6392 (1984)
- 12 Hess P, Lansman JB and Tsien RW: Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature* **311**, 538 – 544 (1984)
- 13 Hering S, Berjukow S, Aczél S and Timin EN: Ca^{2+} channel block and inactivation: common molecular determinants. *Trends Pharmacol Sci* **19**, 439 – 443 (1998)
- 14 Gurney AM, Nerbonne JM and Lester HA: Photoinduced removal of nifedipine reveals mechanisms of calcium antagonist action on single heart cells. *J Gen Physiol* **86**, 353 – 379 (1985)
- 15 Lee KS and Tsien RW: Mechanism of calcium channel blockade by verapamil, D600, diltiazem and nitrendipine in single dialysed heart cells. *Nature* **302**, 790 – 794 (1983)
- 16 Hosono M, Iida H, Ikeda K, Hayashi Y, Dohmoto H, Hashiguchi Y, Yamamoto H, Watanabe N and Yashimoto R: In vitro and ex vivo Ca-antagonistic effect of 2-methoxyethyl(E)-3-phenyl-2-propen-1-yl(±)-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)pyridine-3,5-dicarboxylate (FRC-8653), a new dihydropyridine derivative. *J Pharmacobiodyn* **15**, 547 – 553 (1992)
- 17 Hosono M, Fujii S, Hiruma T, Watanabe K, Hayashi Y, Ohnishi

- H, Takata Y and Kato H: Inhibitory effect of cilnidipine on vascular sympathetic neurotransmission and subsequent vasoconstriction in spontaneously hypertensive rats. *Jpn J Pharmacol* **69**, 127 – 134 (1995)
- 18 Fujii S, Kameyama K, Hosono M, Hayashi Y and Kitamura K: Effect of cilnidipine, a novel dihydropyridine Ca^{++} -channel antagonist, on N-type Ca^{++} channel in rat dorsal root ganglion neurones. *J Pharmacol Exp Ther* **280**, 1184 – 1191 (1997)
- 19 Uneyama H, Takahara A, Dohmato H, Yoshimoto R, Inoue K and Akaike N: Blockade of N-type Ca^{2+} current by cilnidipine (FRC-8653) in acutely dissociated rat sympathetic neurones. *Br J Pharmacol* **122**, 37 – 42 (1997)
- 20 Furukawa T, Yamakawa T, Midera T, Sagawa T, Mori Y and Nukada T: Selectivities of dihydropyridine derivatives in blocking Ca^{2+} channel subtypes expressed in xenopus oocytes. *J Pharmacol Exp Ther* **291**, 464 – 473 (1999)
- 21 Lacinová L and Hofmann F: Isradipine interacts with the open state of the L-type calcium channel at high concentrations. *Receptors Channels* **6**, 153 – 164 (1998)
- 22 Hamill OP, Marty A, Neher E, Sakmann B and Sigworth FJ: Improved patch-clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Eur J Physiol* **391**, 85 – 100 (1981)
- 23 Almers W and Palade PT: Slow calcium and potassium currents across frog muscle membrane: measurements with a vaseline-gap technique. *J Physiol (Lond)* **312**, 159 – 176 (1981)
- 24 Zhang F, Ram JL, Standley PR and Sowers JR: beta-Estradiol attenuates voltage-dependent Ca^{2+} currents in A7r5 vascular smooth muscle cell line. *Am J Physiol* **266**, C975 – C980 (1994)
- 25 Handrock R and Herzig S: Stereoselectivity of Ca^{2+} channel block by dihydropyridines: no modulation by the voltage protocol. *Eur J Pharmacol* **309**, 317 – 321 (1996)
- 26 Handrock R, Rao Schymanski R, Klugbauer N, Hofmann F and Herzig S: Dihydropyridine enantiomers block recombinant L-type Ca^{2+} channels by two different mechanisms. *J Physiol (Lond)* **521**, 31 – 42 (1999)
- 27 Roulet JB, Spaetgens RL, Burlingame T, Feng ZP and Zamponi GW: Modulation of neuronal voltage-gated calcium channels by farnesol. *J Biol Chem* **274**, 25439 – 25446 (1999)
- 28 Bean BP, Cohen CJ and Tsien RW: Lidocaine block of cardiac sodium channels. *J Gen Physiol* **81**, 613 – 642 (1983)
- 29 Lee KS, Marban E and Tsien RW: Inactivation of calcium channels in mammalian heart cells: joint dependence on membrane potential and intracellular calcium. *J Physiol (Lond)* **364**, 395 – 411 (1985)
- 30 Brehm P and Ecker R: Calcium entry leads to inactivation of calcium channel in paramecium. *Science* **202**, 1203 – 1206 (1978)