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Characterization of Ca^{2+} current inhibition by cilnidipine using a β-subunit antisense oligonucleotide

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Abstract

The dihydropyridine cilnidipine has been reported to induce a fast decay of the L-type Ca^{2+} current, an effect distinct from the modulation of voltage-dependent inactivation. We performed a whole-cell patch-clamp study using A7r5 cells to analyse the changes in current decay induced by two structurally related dihydropyridines after the amount of the β-subunit mRNA has been decreased by the antisense oligonucleotide. The Ba^{2+} current underwent a single exponential decay indicating voltage-dependent inactivation. The τ value was greater in the antisense group than in the sense and nonsense groups. Equipotent doses of cilnidipine and nimodipine decreased the τ value, while only cilnidipine created an additional component with a smaller τ value (τ_{cil}: 35 ms) that showed voltage-independence. Treatment with antisense failed to alter this component (τ_{cil}: 38 ms). These results suggest that cilnidipine specifically exerts a second type of blocking action on L-type Ca^{2+} channels that is not dependent on the β-subunit.

Keywords: Ca^{2+} channel, L-type; Dihydropyridine; Cilnidipine; Antisense; β-Subunit; A7r5 cell

1. Introduction

Dihydropyridines are Ca^{2+} channel blockers that are important therapeutically in the treatment of cardiovascular disorders. The mechanism by which dihydropyridines interact with Ca^{2+} channels has been interpreted as showing state-dependent binding of these compounds to their receptors (Mcdonald et al., 1984; Uehara and Hume, 1985; Hering et al., 1988). On the basis of the traditional three-state model (resting, open and inactivated states), dihydropyridines are said to act preferentially on the inactivated state (Kass and Sanguinetti, 1984; Bean, 1984). Several approaches, such as photoaffinity labelling and site-directed mutagenesis, have now revealed dihydropyridine binding sites in the IIIS6 and IVS6 segments of the α_1-subunit of the L-type Ca^{2+} channel (Nakayama et al., 1991; Striessnig et al., 1998). In these segments of the α_1-subunit, several amino acids have been identified as inactivation determinants and some are thought to serve as high-affinity determinants for the dihydropyridine binding site (Hering et al., 1998).

It is notable that a different type of inhibition has also been reported, in which dihydropyridines cause an acceleration of the current decay seen during step pulses (Hess et al., 1984; Hering et al., 1998; Gurney et al., 1985; Lacinová and Hofmann, 1998). Recently, we reported that cilnidipine (Fig. 1), a long-acting antihypertensive compound (Hosono et al., 1992, 1995), possesses a potent current-decaying action, an action not shared by the structurally related dihydropyridine nimodipine (Uchida et al., 2001). In that study, we concluded that cilnidipine probably exerts this blocking action on the open state of the channel. However, it is difficult to distinguish an action on the open state from one on the inactivated state, since these two phenomena take place concurrently during depolarising voltage steps.

Much evidence has accumulated to indicate that auxiliary subunits confer specific physiological characteristics on the Ca^{2+} channel, with the α_1-subunit, forming the pore region of the channel, being indispensable for channel function (Varadi et al., 1991; Singer et al., 1991). Among the auxiliary subunits, the β-subunit plays a pivotal role in modulating channel kinetics, such as inactivation (Singer et al., 1991; Varadi et al., 1991; Lory et al., 1993). Therefore, it

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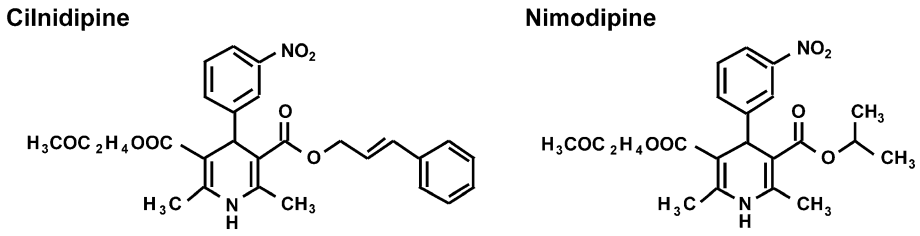

Fig. 1. Chemical structures of cilnidipine and nimodipine. Both of the compounds employed in the present study were in the racemic form.

seems reasonable to postulate that manipulating the β-subunit would modify the effectiveness of Ca^{2+} channel blockers by altering channel kinetics. In fact, in Chinese hamster ovary (CHO) cells coexpressing α_{1C}- and β_3-subunits (which accelerated current inactivation), another type of Ca^{2+} channel blocker, verapamil, has been reported to block the Ca^{2+} current to a greater extent than in CHO cells expressing the α_{1C}-subunit alone (Lacinová et al., 1995). Recently, cilnidipine has been reported to be more potent at blocking the Ba^{2+} current in A7r5 cells expressing multi-subunits than in *Xenopus* oocytes expressing the α_{1C}-subunit alone (Löhn et al., 2002). Hence, it seems likely that depletion of the β-subunit would disclose whether a given dihydropyridine has an action that is dependent on the open state in addition to its action on the inactivated state.

In the present report, we focused firstly on the role of the β-subunit in the activity of L-type Ca^{2+} channels in A7r5 cells, a cell-line derived from the rat thoracic aorta. To this end, we decreased the amount of the β-subunit mRNA in these cells by transfecting the antisense oligonucleotide of the subunit. This procedure can be expected to at least alter the kinetics of the inactivated state and concurrently any drug action that is dependent on the inactivated state. Having confirmed suppression of the amount of β-subunit protein by immunocytochemistry, we performed a conventional whole-cell patch-clamp study using these cells (as well as cells transfected with nonsense or sense oligonucleotides). Thereafter, we attempted to distinguish two types of current inhibition by both cilnidipine and nimodipine by analysing the kinetics of the current decay and by examining the steady-state blockade.

2. Materials and methods

2.1. Cell culture

The A7r5 cell line, derived from the rat thoracic aorta, was obtained from the American Type Culture Collection (Rockville, MD, USA). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Dainippon Pharm., Osaka, Japan) in the presence of 10% heat-inactivated (56 °C, 40 min) fetal calf serum (ICN Biomedicals, OH, USA), 1.7 mM L-glutamine, 50 μM gentamicin sulphate (Gibco, NY, USA) and nonessential 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, Japan) placed in a tissue-culture dish or flask (Becton Dickinson, USA) at a density of 2.0×10^3 cells cm^{-2}.

2.2. Oligonucleotides

Phosphorothioate deoxyoligonucleotides were used in the present study. The β-subunit antisense oligonucleotide had the sequence: 5′ACCAGCCTTCCGATCCACCA-GTCATT3′ (synthesized by Amersham Pharmacia Biotech, Tokyo, Japan). This sequence is 96% complementary to the β_{1b} mRNA and 92% complementary to the β_2, β_3 and β_4 mRNAs (Berrow et al., 1995). The nonsense oligonucleotide had the sequence: 5′GAAGTAGGTCTTGGTG-GTGG3′, which has no target sequence of significant homology within existing databases. The sequence of the sense oligonucleotide was 5′AATGACTGGTGGATCGGA-AGGCTGGT3′.

The cells were cultured for 5 days before transfection with oligonucleotides. A given oligonucleotide and the transfection reagent Tfx-50 (Promega, Madison, WI, USA) were dissolved in serum-containing medium and incubated at room temperature for 10–15 min. The oligonucleotide-Tfx-50 mixture was then added to A7r5 cells in a 35-mm dish [or a culture plate (24 wells) in the case of the immunocytochemical experiments], followed by a 1-h incubation at 37 °C. The final oligonucleotide concentration was 1.5 μM. At the end of the incubation, supplementary DMEM (twice the volume of the incubation mixture) was added to the dish. The cells were thereafter cultured for 48 h before the patch-clamp experiment or immunocytochemistry.

2.3. Immunocytochemistry

A7r5 cells (ca. 4×10^8 cells) in a tissue-culture plate were washed with Ca^{2+}- and Mg^{2+}-free phosphate buffer (PBS; 137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4), fixed with 4% paraformaldehyde in PBS for 1 h at 4 °C and permeabilized with 0.02% Triton X-100 for 30 min. The cells were washed (4×5 min) and incubated with 1.5% goat serum for 30 min at room temperature. They were then incubated overnight at 4 °C in PBS containing rabbit anti-Ca^{2+} channel β_3-subunit antibody (dilution, 1:400; ACC-008, Alomone Labs, Israel) together with 0.025% NaN_3, 0.05% Tween-20 and 3%

bovine serum albumin. In some experiments, the antibody was pre-absorbed by antigen peptide to produce a negative control. The cells were washed (4 × 5 min) and incubated for 1 h at room temperature with a 1:800 dilution in PBS of goat antirabbit immunoglobulin G conjugated with biotin. Thereafter, the cells were washed (4 × 5 min) and incubated for 10 min with an avidin-biotinylated horseradish peroxidase complex (Vectastain ABC kit; Vector Lab., Burlingame, USA). Immunostaining was performed using 3,3′-diaminobenzidine in imidazole buffer containing H_2O_2 (Dako, Carpinteria, USA).

2.4. Electrophysiology

The transfected or nontransfected 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 ml min^{-1}. Currents were recorded via an EPC-9 amplifier (HEKA elektronik, Lambrecht/Pfalz, Germany) in a whole-cell configuration using a conventional patch-clamp technique (Hamill et al., 1981). Electrode pipettes were made from borosilicate glass capillaries (1.5 mm OD; Narishige Scientific Instrument Lab, Tokyo, Japan) using a Flaming-Brown micropipette puller (model P-87; Sutter Instruments, CA, USA), and were then heat-polished (MF-83; Narishige). Their resistance was 2–4 MΩ 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 on-line at 20 kHz using a Power Macintosh G3 DT266 (Apple Computer, CA, USA). All experiments were conducted at room temperature (22–25 °C).

2.5. Solutions

To measure Ba^{2+} currents during whole-cell recording, A7r5 cells were perfused with a bathing solution containing (in mM): 162 tetraethylammonium chloride, 10 $BaCl_2$, 10 glucose and 10 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulphonic acid (HEPES). Patch pipettes were filled with a solution containing (in mM): 125 CsOH, 125 L-aspartic acid, 5 1,2-bis(2-aminophenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA), 10 HEPES, 5 ATP·2 Na salt and 5 $MgCl_2$. The pH of the bathing solution was adjusted to 7.4 using tetraethylammonium hydroxide and that of the pipette solution to 7.2 using CsOH.

2.6. Chemicals

The chemical structures of cilnidipine and nimodipine are shown in Fig. 1. Cilnidipine (synthesized by Fuji-Rebio, Tokyo, Japan) and nimodipine (Calbiochem, CA, USA) were dissolved in dimethylsulfoxide (DMSO; Sigma) 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 Ba^{2+} current recorded in A7r5 cells. These compounds were applied by superfusion to the bath. The action of cilnidipine was slow, especially at low concentrations (<100 nM). Since a rundown of the Ba^{2+} current was observed in our experiments, we felt we could not wait for more than 10 min to measure the current following drug application. In the present study, therefore, we measured the current 5–10 min after application of cilnidipine at a concentration of as much as 1 μM. 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. Other compounds were obtained from Sigma.

2.7. Data analysis

Capacitative and leak currents were eliminated using the P/4 method (Almers and Palade, 1981). 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 effect achieved after application of the relevant drug. Data were analysed using the PulseFit program (HEKA elektronik). Curve-fitting was carried out using Igor Pro (WaveMetrics, OR, USA) and Kaleida Graph (Synergy Software, PA, USA) programs.

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

$$I(t) = A\exp(-t/\tau) + C \text{ or}$$

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

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 nondecaying component.

Steady-state inactivation curves were fitted using the Boltzman equation. The relative amplitude of the L-type current (I) observed at one of a number of voltages (V) of prepulse was expressed as:

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

where I_{max} is the current amplitude without a prepulse, V_{half} is 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 noninactivating component.

2.8. 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 two groups were to be compared or by a one-way analysis of variance (ANOVA) followed by a post-hoc Bonferroni's *t*-test for more than three groups. The dependence of τ on the voltage of the depolarizing pulse was analysed by comparison between τ values at -10 and $+30$ mV using a paired *t*-test. A *P*-value less than 0.05 was considered to be statistically significant.

3. Results

A7r5 cells have been reported to express two distinct types of Ca^{2+} channel (L- and T-types) (Zhang et al., 1994). In preliminary experiments, we examined the current–voltage relationship of the Ca^{2+} channels in A7r5 cells and found that most cells exhibited an inward current with a high-voltage threshold (Uchida et al., 2001). At 10 μM, cilnidipine and nifedipine each completely inhibited that current (probably corresponding to the L-type current). In the present study, all of the cells tested exhibited only a dihydropyridine-inhibitable inward current with a high-voltage threshold (data not shown) and so we concluded that the current recorded was mainly of the L-type.

3.1. Suppression of β-subunit expression by antisense oligonucleotide

Using a commercially available anti-β_3-subunit antibody, we examined first the expression of the β_3-subunit by immunostaining using nontreated A7r5 cells and cells transfected with antisense, sense or nonsense oligonucleotides. The antibody against the β_3-subunit stained the nontreated cells and the sense- and nonsense-transfected cells, but not the antisense-transfected cells (Fig. 2). Pre-absorption of the antibody by control antigen peptide diminished the staining (nontreatment + antigen and antisense + antigen). This suggests that the present antisense procedure at least decreased the amount of the β_3-subunit, one of the β-subunits known to be expressed in vascular smooth muscle cells (Hullin et al., 1992) and A7r5 cells (Bielefeldt, 1999).

Next, we tested the validity of the present antisense procedure by comparing current–voltage relationships among A7r5 cells transfected with different oligonucleotides. Fig. 2 shows representative traces of Ba^{2+} currents obtained during voltage-ramps from -80 to $+80$ mV. In cells treated with antisense, the peak current (I_{peak}) was significantly smaller (76.8 ± 18.5 pA, $n=3$) than that observed in either the sense group (338.8 ± 21.8 pA, $P<0.001$, $n=3$) or the nonsense group (277.2 ± 56.2 pA, $P<0.001$, $n=3$). The membrane potential that gave I_{peak} was shifted in the positive direction by antisense (antisense, 30.0 ± 0.9 mV, $n=3$; sense, 8.7 ± 2.1 mV, $P<0.01$, $n=3$; nonsense, 9.6 ± 1.5 mV, $P<0.05$, $n=3$). These results imply that the present procedure alters the properties of the Ca^{2+} channel, as previously reported (Berrow et al., 1995), and are in accordance with the present immunocytochemistry data obtained using the anti-β_3-subunit antibody.

3.2. Cilnidipine-specific effect on Ca^{2+} current decay in A7r5 cells

To examine the time course of the Ca^{2+} current, the holding potential was set at -80 mV and step pulses to 10 mV were applied for 1 s every 15 s. A pulse duration of 1 s seemed to be appropriate for an analysis of the kinetics

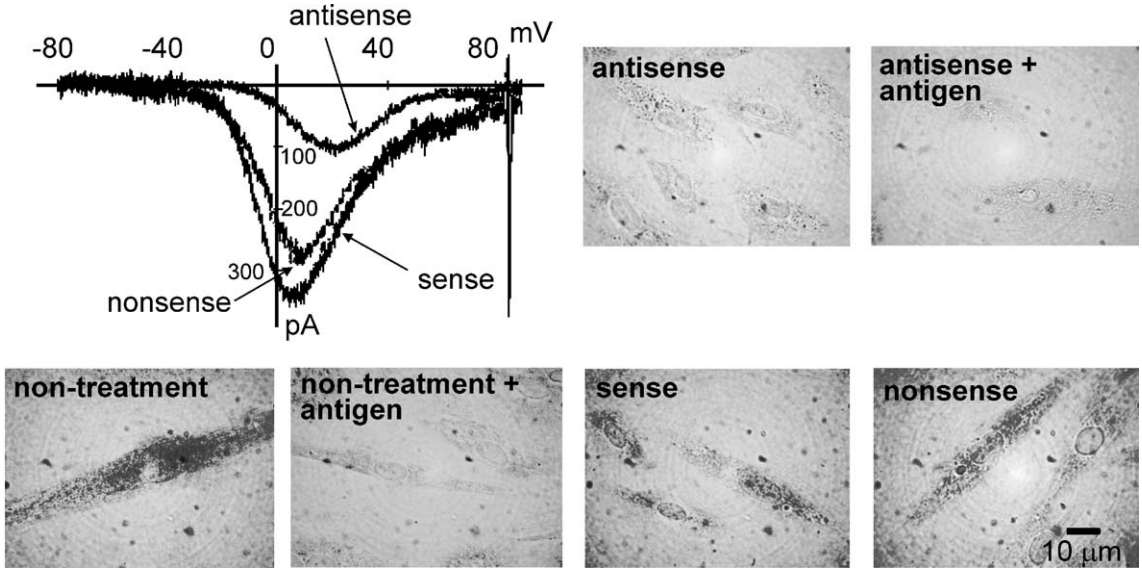

Fig. 2. Typical traces of L-type current evoked by voltage-ramp from -80 to 80 mV (300 ms) recorded in A7r5 cells transfected with sense, nonsense or antisense oligonucleotides. Immunostaining for β_3-subunit of L-type Ca^{2+} channels is also shown in A7r5 cells either nontreated or treated with antisense, nonsense or sense oligonucleotides. An anti-β_3-subunit antibody was used. Nontreatment + antigen, antisense + antigen; the antibody was pre-absorbed by control antigen peptide.

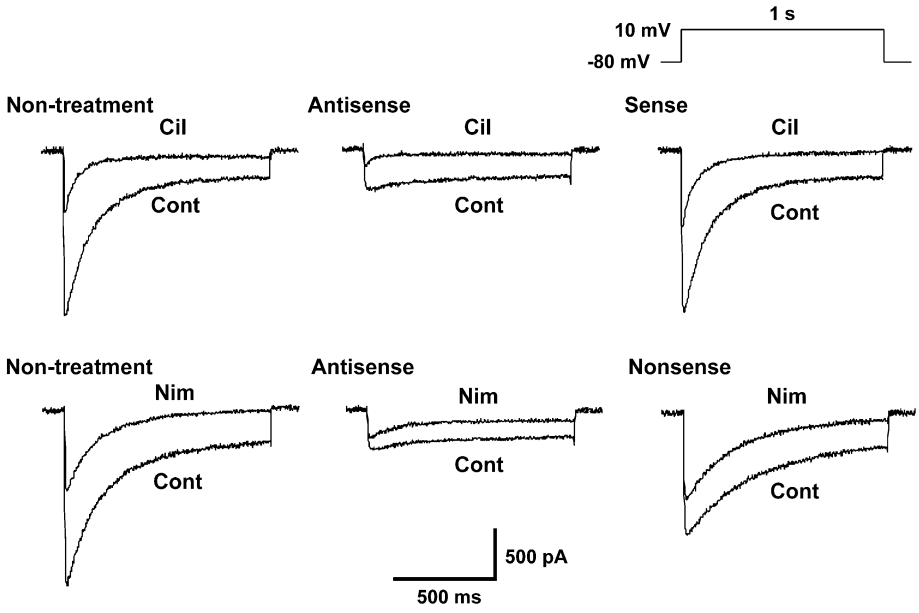

Fig. 3. Effects of 1 μM cilnidipine and 100 nM nimodipine on L-type current in A7r5 cells either nontreated or transfected with sense, nonsense or antisense oligonucleotides. Superimposed current traces were obtained before (Cont) and after application of cilnidipine (Cil) or nimodipine (Nim). Ba^{2+} current was evoked by depolarising pulses to +10 mV for 1 s from a holding potential of −80 mV.

of current decay ($\tau < 300$ ms) and the interval between steps should have been long enough for the channels to escape from the inactivated state. As also found in the experiment using the ramp protocol performed (see above), I_{peak} was significantly smaller in the antisense group (192.7 ± 10.6 pA, $n = 14$) than in the other groups [non-treated group (492.3 ± 22.2 pA, $n = 38$, $P < 0.05$), sense group (586.3 ± 6.7 pA, $n = 3$, $P < 0.01$), nonsense group (427.7 ± 26.6 pA, $n = 6$, $P < 0.05$)].

Fig. 3 shows representative Ba^{2+} current traces recorded with or without the presence of Ca^{2+} channel blockers. In the nontreated group, cilnidipine 1 μM inhibited the peak current (I_{peak}) by 62.7 ± 12.7% ($n = 9$), while nimodipine 100 nM inhibited it by 57.2 ± 19.8% ($n = 5$), suggesting that the inhibitory effects of these Ca^{2+} channel blockers are almost equipotent at these doses. We therefore employed these compounds at the above concentrations in the subsequent experiments. When cilnidipine (1 μM) was applied to cells in the nonsense and sense groups, I_{peak} was inhibited to about 50% of the value recorded before its application, while in the antisense group I_{peak} was reduced to about 40% of the predrug value (Fig. 4).

To compare time-dependent inhibitions of the Ba^{2+} current induced by the two dihydropyridines, we quantified the current amplitude at 50 ms (I_{50}) during step pulses to obtain a sensitive indication of any change in a current component with a smaller τ (Fig. 4). The inhibition of I_{50} by cilnidipine was significantly greater than its inhibition of

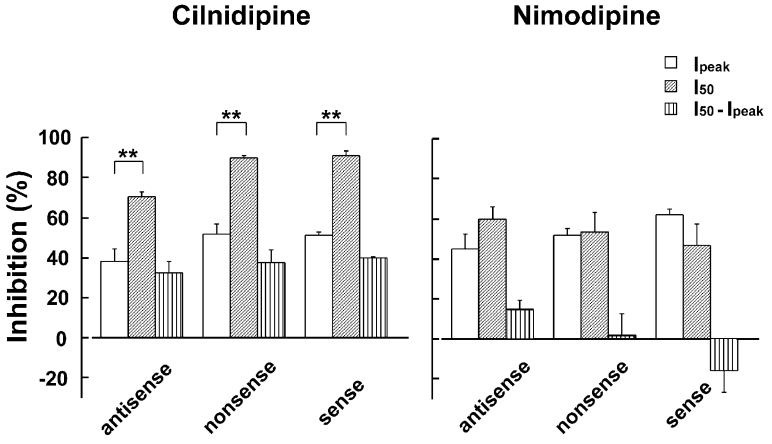

Fig. 4. Inhibition of L-type current by 1 μM cilnidipine and 100 nM nimodipine in A7r5 cells with or without treatment with oligonucleotides: antisense, nonsense and sense. Ba^{2+} current was evoked by depolarising pulses to +10 mV for 50 ms from a holding potential of −80 mV. Percentage inhibition is expressed in two different ways, either from the peak amplitude (I_{peak}) or from the amplitude at 50 ms during step pulses (I_{50}). The difference between the two values ($I_{50} - I_{peak}$) is also shown. $^{**}P < 0.01$, I_{peak} versus I_{50}.

I_{peak} in the antisense group, and the same was true for the nonsense and sense groups. There was no statistical difference among the difference values between I_{peak} and I_{50} ($I_{50} - I_{peak}$) obtained for the three groups ($P>0.05$, multiple t-test), suggesting that cilnidipine accelerated current decay in each case. By contrast, nimodipine (100 nM) exhibited a strikingly different pattern of channel modulation in that in each group, this compound inhibited I_{peak} and I_{50} to a similar degree (I_{peak} to 45–62% and I_{50} to 46–60% of the predrug values) (Fig. 4). With nimodipine, there was no statistical difference between I_{peak} and I_{50} in any group and thus the values obtained for $I_{50} - I_{peak}$ were all close to zero.

3.3. Cilnidipine-induced current decay in A7r5 cells transfected with oligonucleotides

In the absence of any drug, it was notable that current decay appeared to be slower in the antisense group (Fig. 3). In an attempt to quantify this, the decay kinetics were estimated from current traces recorded during 1-s step pulses. Previously, we reported that the current decay can be well fitted to a single exponential function (Table 1, nontreatment; Uchida et al., 2001). The estimated τ value was within the hundreds of milliseconds range (187–288 ms) and was significantly greater in the antisense group than in the nonsense and sense groups (Table 1, control). The fractional size of the component was significantly less in the antisense group than in the other groups. The presence of a large residual component after exponential fitting in the antisense group (53.6%) would imply an extremely slow inactivation of the channel with a larger time constant (Kass and Sanguinetti, 1984). In our previous paper (Uchida et al., 2001), we concluded that the presence of a component with a time constant of several hundred milliseconds reflects a process of voltage-dependent inactivation of the Ca^{2+} channel. Indeed, Fig. 5 (open circles) clearly shows that a greater depolarization leads to a smaller τ value (i.e., faster inactivation). Therefore, a decrease in the amount of the β-subunit seems to modify the voltage-dependent inactivation process.

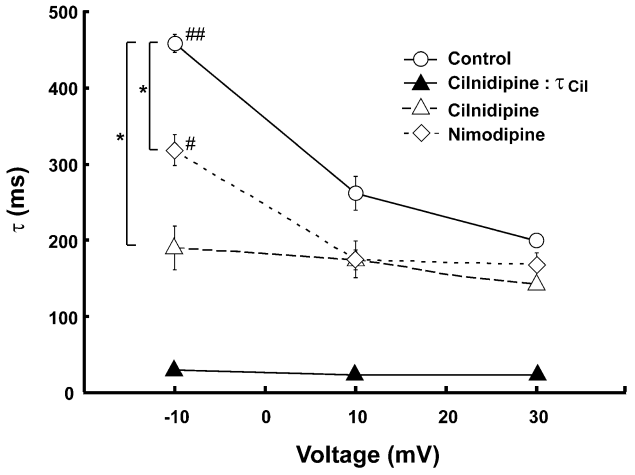

Fig. 5. Relationships between time constant of current decay and the voltage of the depolarising pulse in the absence and presence of cilnidipine or nimodipine. A7r5 cells were transfected with β-subunit antisense oligonucleotide. The Ba^{2+} current was evoked by depolarising pulses to three different potentials (−10, 10 and 30 mV) for 1 s from a holding potential of −80 mV. The currents were fitted with single (control and nimodipine) or double exponential functions (cilnidipine) (see Section 2.7 in Section 2). τ_{Cil} indicates the smaller time constant that appeared only in the presence of cilnidipine. Each data-point represents mean ± S.E.M. for five observations (control), three observations (cilnidipine) or four observations (nimodipine). $^{*}P<0.05$, control versus cilnidipine or nimodipine at −10 mV. $^{\#}P<0.05$, $^{\#\#}P<0.01$, −10 versus +30 mV.

When cilnidipine was applied to cells in the nonsense, sense and nontreated groups, the current decay was greatly accelerated (raw traces for the nonsense, sense and nontreated groups are shown in Fig. 3). The current trace recorded in its presence was not well fitted to a single exponential function but to the sum of two exponential functions. The faster τ value (τ_{Cil}) was 35 ms and the slower one 212–218 ms (Table 1, cilnidipine). Cilnidipine significantly reduced the slower τ value in the antisense group at

Table 1
Effects of oligonucleotides on decay of L-type Ba^{2+} current

Drugs	Oligonucleotide	n	τ_{Cil} (ms)	Fraction (%)	τ (ms)	Fraction (%)	Residual Fraction (%)
Control	antisense	34			288 ± 20	46.4 ± 1.9	53.6 ± 1.9
	nonsense	24			234 ± 17	76.4 ± 1.6	23.6 ± 1.6
	sense	16			187 ± 16	78.1 ± 2.1	21.9 ± 2.1
	non-treatment[a]	78			195 ± 7	84.0 ± 1.4	16.0 ± 1.4
Cilnidipine 1 μM	antisense	13	38 ± 5	54.1 ± 3.4	214 ± 37	26.8 ± 3.0	19.2 ± 1.1
	nonsense	14	35 ± 3	57.7 ± 3.4	218 ± 18	32.5 ± 3.9	9.9 ± 1.7
	sense	11	35 ± 2	54.2 ± 4.5	212 ± 30	34.0 ± 3.9	11.8 ± 3.9
	non-treatment[a]	34	37 ± 2	63.1 ± 3.2	202 ± 23	30.4 ± 2.2	6.6 ± 1.8
Nimodipine 100 nM	antisense	16			234 ± 39	56.2 ± 5.0	43.8 ± 5.0
	nonsense	6			178 ± 33	87.7 ± 2.2	12.3 ± 2.2
	sense	6			103 ± 6	93.0 ± 2.7	7.0 ± 2.7
	non-treatment[a]	16			141 ± 8	92.6 ± 1.2	7.4 ± 1.2

Ba^{2+} current was evoked by a 1-s depolarising pulse to 10 mV from a holding potential of −80 mV. $^{*}P<0.05$, $^{**}P<0.01$.
[a]Data for nontreated group are from our previous paper (Uchida et al., 2001).

− 10 mV (Fig. 5). In contrast to the situation in the control groups, under cilnidipine the slower τ value in the antisense group appeared not to be different from those in the nonsense and sense groups (Table 1) and to exhibit little or no voltage-dependence (Fig. 5). Possibly, this may be because the estimation of τ in the presence of cilnidipine was limited by this component making up a smaller fraction (26–34%) of the total current, in addition to the amplitude of the current being smaller (40% of that in the nontreated group).

The τ_{Cil} component appeared only in the presence of cilnidipine, having not been detected in any of the control groups (Table 1). Our previous report showed that the appearance of a faster component reflects the open-channel blocking action of this compound (Uchida et al., 2001). Fig. 5 (closed triangles) clearly shows that τ_{Cil} failed to exhibit voltage-dependency. This result argues against the involvement of τ_{Cil} in the voltage-dependent inactivation process. It is notable that when cilnidipine (1 μM) was applied to cells in the antisense group, the faster component was still present, the τ_{Cil} value being comparable to those obtained for the nonsense, sense and nontreated groups (38 versus 35 ms, Table 1).

In the presence of an equipotent dose of nimodipine (100 nM), the decaying current trace could be well fitted solely to a single exponential function in the antisense, nonsense, sense and nontreated groups with τ values of 103–234 ms (Table 1, nimodipine). Nimodipine significantly reduced the τ value in the antisense group at − 10 mV (Fig. 5). In the antisense group, the τ value was larger than that obtained for the sense group and was voltage-dependent (Fig. 5). The fractional size of this component was significantly smaller in the antisense group than in the other groups (Table 1). A faster decaying component, however, was not evident in any group in the presence of nimodipine, only in the presence of cilnidipine (Table 1).

3.4. Steady-state inactivation curves for the L-type current

Next, we examined the effects of depleting the β-subunit on the modification of the inactivated state by cilnidipine and nimodipine. A long prepulse (3 s) at various potentials was applied every 15 s just before the test pulse (to + 10 mV, 50 ms in duration). As shown in Fig. 6, in the antisense group the decrease in the amplitude of the Ba^{2+} current evoked by the test pulse became greater as the prepulse potential was increased in the depolarising direction. A striking feature shown by the antisense group was a noninactivating component (35% of the total) at positive potentials up to + 10 mV (open circles). This phenomenon was not seen at such potentials in the nontreated group in our previous report (see the thinner solid line in the present Fig. 6; Uchida et al., 2001). Perfusion with 1 μM cilnidipine or 100 nM nimodipine, which produced equipotent inhibitions of the peak Ba^{2+} current, abolished this noninactivating component at potentials of more than 0 mV (Fig. 6), suggesting that these compounds can modify inactivation

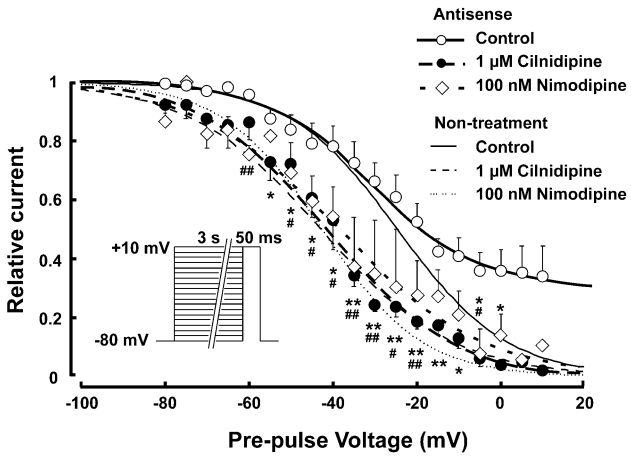

Fig. 6. Steady-state inactivation curves for L-type current obtained in the absence or presence of drug (cilnidipine or nimodipine) in A7r5 cells transfected with β-subunit antisense oligonucleotide. Voltage protocol is indicated in the inset. Relative amplitude of the Ba^{2+} current evoked by a test pulse (to 10 mV, 50 ms duration) is plotted against the amplitude of the prepulse 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 normalised as 1.0. Each symbol indicates mean ± S.E.M. (control, $n=4$; cilnidipine, $n=5$; nimodipine, $n=4$). The theoretical curves were drawn using the Boltzman equation (see Section 2). The estimated parameters are as follows: (control) $V_{half}=-30.6\pm0.7$ mV, $k=-13.0\pm0.6$ mV; (cilnidipine) $V_{half}=-40.8\pm1.3$ mV, $k=-14.4\pm1.2$ mV; (nimodipine) $V_{half}=37.9\pm1.2$ mV, $k=-17.2\pm1.2$ mV. $^{*,\#}P<0.05$, $^{**,\#\#}P<0.01$, versus control value at the same potential (asterisk, 1 μM cilnidipine; hatch, 100 nM nimodipine). Thinner solid, broken and dotted lines show the steady-state inactivation curves obtained from nontransfected A7r5 cells (non-treatment) in our previous report (Uchida et al., 2001).

profiles that are dependent on the β-subunit. These inhibitors also markedly shifted the steady-state inactivation curve to the left by ca. 10 mV (Fig. 6). The relative currents obtained in the presence of these compounds were significantly smaller than those obtained in their absence at each prepulse voltage. The curves obtained in the absence and presence of either cilnidipine or nimodipine (Fig. 6) were used to provide V_{half} values. There was no significant difference between the shifts in V_{half} produced by these two blockers at these concentrations ($P>0.05$) and the k values were not different among the three conditions ($P>0.05$).

4. Discussion

We previously reported that cilnidipine exhibits a second type of Ca^{2+} channel-blocking action (viz. acceleration of the decay of the L-type Ca^{2+} current) in addition to the channel inhibition elicited preferentially in the inactivated state that is shared by other dihydropyridines, like nimodipine (Uchida et al., 2001). In the present study, by transfecting a β-subunit antisense oligonucleotide, we demonstrated that the enhancement of current decay induced by cilnidipine is observed even if the β-subunit

is decreased and is entirely distinct from the action of this drug on the inactivated state (which can be modified by a depletion of the β-subunit).

Among the four types of β-subunits (β_1, β_2, β_3 and β_4) identified in many tissues, the β_2- (Perez-Reyes et al., 1992) and β_3-subunits (Castellano et al., 1993) have been reported to be present in the rat aorta (Hullin et al., 1992). Bielefeldt (1999) has shown that β_{1b}, β_2 and β_3 can be found in A7r5 cells. In the present study, using a commercially available anti-β_3-subunit antibody, we examined the expression of the β_3-subunit by immunoperoxidase-labelling using nontreated cells and cells transfected with antisense, sense and nonsense oligonucleotides. The cells in the nontreated, sense and nonsense groups were stained, while staining was faint in the antisense group, suggesting that the present antisense procedure decreased the β_3-subunit at least.

In the present study, a decrease in amount of the β-subunit by oligonucleotide transfection altered I_{peak}, the membrane potential giving I_{peak}, the inactivation kinetics and the steady-state inactivation curve of the Ca^{2+} current. One of the important functions of the β-subunit is to modify the biophysical properties of Ca^{2+} channels, as shown in coexpression studies using *Xenopus* oocytes and mammalian cells (Singer et al., 1991; Varadi et al., 1991; Itagaki et al., 1992; Lory et al., 1993; Cens et al., 1999). Previous studies have also revealed that depletion of the β-subunit by antisense oligonucleotides reduces the amplitude and/or slows the inactivation kinetics of the Ca^{2+} current (Berrow et al., 1995; Kimura et al., 2000). These results obtained in antisense studies seem to correlate well with the previous results obtained from heterologous coexpression of cloned β- and α_1-subunits (Singer et al., 1991; Varadi et al., 1991; Itagaki et al., 1992; Lory et al., 1993; Cens et al., 1999). Molecular biology experiments have revealed direct interactions between the various subunits. For instance, the β-subunit has been reported to interact noncovalently with a specific binding site, designated the alpha interaction domain, located in the intracellular loop between transmembrane repeats I and II (Pragnell et al., 1994). On the other hand, several lines of evidence showing that the I–II loop modulates inactivation have been obtained using the following techniques: mutation of the I–II loop of the α_1-subunit, construction of a chimera by replacing the I–II loop and coexpression with an excess of free I–II loop peptide (Adams and Tanabe, 1997; Herlitze et al., 1997; Sokolov et al., 1999; Berrou et al., 2001). Thus, our results support the idea that coexpression of the β-subunit confers specific biophysical characteristics on the channel. If this is so, introducing a β-subunit antisense oligonucleotide may prove to be a useful way to alter the inactivation kinetics of the channel.

The antisense, sense and nonsense groups all exhibited slow τ values (a few hundred milliseconds) in the presence or absence of cilnidipine and nimodipine (Table 1). This decay appeared to represent the time course of voltage-dependent inactivation of the channel since the values were larger at more negative potentials, as previously documented (the present Fig. 5; Kass and Sanguinetti, 1984; Lee et al., 1985). Both cilnidipine and nimodipine reduced the slow τ values, particularly at negative potentials (viz. they accelerated the inactivation of the channel) (Uchida et al., 2001). This ability to affect inactivation kinetics is in line with the idea that these dihydropyridines favour the inactivated state of L-type Ca^{2+} channels lacking the β-subunit as well as that of the native channel (Kass and Sanguinetti, 1984; Bean, 1984). These dihydropyridines can modify inactivation profiles that are dependent on the β-subunit, since they abolished the noninactivated component that was observed only in the antisense group (Fig. 6). The notion of a relation between the affinity of dihydropyridine binding site and the β-subunit is supported by the recent finding that mutation of the β-subunit reduces [^{3}H]isradipine binding affinity (Hitzl et al., 2002).

The present study clearly demonstrated that a fast current decay ($\tau = 35$ ms) was present de novo under cilnidipine (Table 1). The finding that a decrease in the amount of the β-subunit failed to alter the kinetics and fractional size of the fast decay seems to be supporting evidence for the idea that the change in current decay due to cilnidipine has little relation to the action of the compound on the inactivated state, since the β-subunit is known to alter the inactivation properties of the channel (see above discussion). In fact, an acceleration of current decay by certain dihydropyridines has previously been documented (Lee and Tsien, 1983; Hess et al., 1984; Gurney et al., 1985; Hering et al., 1998; Lacinová and Hofmann, 1998). Recently, we showed that the fast decay seen under cilnidipine can be interpreted as an open-channel blocking effect on the basis of a simple receptor–drug interaction (Uchida et al., 2001). The interesting finding in the present report is that an enhancement of Ca^{2+} current decay is likely to require a specific compound structure. Cilnidipine clearly induced a fast current decay, while nimodipine, a structurally related compound, failed to alter the kinetics of the Ca^{2+} current. A structural selectivity of drug action is generally considered to reflect the structure of the binding site and the interaction between drug and binding site. This suggests that cilnidipine may bind to a specific type of receptor that is distinct from the receptors that favour the inactivated state in their binding affinity (which are shared by all dihydropyridines). Taking all of these findings together suggests to us that cilnidipine is likely to induce a faster current decay due to a second type of blocking action (probably open-channel blockade) that is not dependent on the presence of the β-subunit, rather than through its action on the inactivated state (which is readily affected by transfection of a β-subunit antisense oligonucleotide).

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