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Dihydropyridine Ca^{2+} channel antagonists and agonists block Kv4.2, Kv4.3 and Kv1.4 K^+ channels expressed in HEK293 cells

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1 We have determined the molecular basis of nicardipine-induced block of cardiac transient outward K^+ currents (I_{to}). Inhibition of I_{to} was studied using cloned voltage-dependent K^+ channels (Kv) channels, rat Kv4.3L, Kv4.2, and Kv1.4, expressed in human embryonic kindey cell line 293 (HEK293) cells.

2 Application of the dihydropyridine Ca^{2+} channel antagonist, nicardipine, accelerated the inactivation rate and reduced the peak amplitude of Kv4.3L currents in a concentration-dependent manner (IC_{50}: 0.42 μM). The dihydropyridine (DHP) Ca^{2+} channel agonist, Bay K 8644, also blocked this K^+ current (IC_{50}: 1.74 μM).

3 Nicardipine (1 μM) slightly, but significantly, shifted the voltage dependence of activation and steady-state inactivation to more negative potentials, and also slowed markedly the recovery from inactivation of Kv4.3L currents.

4 Coexpression of K^+ channel-interacting protein 2 (KChIP2) significantly slowed the inactivation of Kv4.3L currents as expected. However, the features of DHP-induced block of K^+ current were not substantially altered.

5 Nicardipine exhibited similar block of Kv1.4 and Kv4.2 channels stably expressed in HEK293 cells; IC_{50}'s were 0.80 and 0.62 μM, respectively.

6 Thus, at submicromolar concentrations, DHP Ca^{2+} antagonist and agonist inhibit Kv4.3L and have similar inhibiting effects on other components of cardiac I_{to}, Kv4.2 and Kv1.4.
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Abbreviations: DHP, dihydropyridine; HEK293, human embryonic kidney cell line 293; I_A, A-type K^+ current; IC_{50}, concentration required for 50% inhibition; I_{to}, transient outward current; KChIP, potassium channel-interacting protein; K_d, dissociation constant; Kv, voltage-dependent K^+ channels

Introduction

The activity of voltage-dependent K^+ (Kv) channels regulates the threshold, shape, duration, and frequency of action potentials in a wide variety of excitable cells. A Ca^{2+}-independent transient outward K^+ current, I_{to}, makes an important contribution to the early repolarization phase of the cardiac action potential (Nerbonne, 2001), and is expressed in most regions of the heart in different density including atrium, ventricle, sinoatrial and atrioventricular nodes, and Purkinje fibers of several species (Campbell *et al.*, 1995; Han *et al.*, 2002). I_{to} density is more prominent in the atrium than in the ventricle of the rat, rabbit, and human heart (Giles & Imaizumi, 1988; Boyle & Nerbonne, 1992; Varro *et al.*, 1993), although regional differences in I_{to} density have been observed across the wall of the left ventricle. This results in the regional differences in action potential morphology (Dixon & McKinnon, 1994; Brahmajothi *et al.*, 1999).

Many time- and voltage-dependent Kv channels, which contribute to the J-wave of the electrocardiogram, have been cloned from the mammalian myocardium, and this approach and other molecular biological techniques have identified the candidates for K^+ channels responsible for I_{to}, (Pongs, 2001). For example, Fiset *et al.* (1997) have shown with antisense oligodeoxynucleotides that Kv4.2 and Kv4.3 contribute to I_{to} in rat ventricular myocytes. It has also been shown that Kv4 members contribute to I_{to} in murine atrial myocytes using a dominant-negative mutant targeted against Kv4 (Barry *et al.*, 1998; Xu *et al.*, 1999). The experiments with antisense oligodeoxynucleotides have also shown that Kv4.3 alone is a major component of I_{to} in human and canine heart, whereas a combination of three cloned channels, Kv4.2, Kv4.3, and Kv1.4, is responsible for I_{to} in rabbit atrium, suggesting that physiological differences between human and rabbit I_{to} may reflect differential expression of K^+ channel α subunit genes (Kong *et al.*, 1998; Wang *et al.*, 1999).

Dihydropyridines (DHPs), such as nifedipine and nicardipine, are widely used for the treatment of cardiovascular disorders, which are considered to be selective blockers of L-type voltage-dependent Ca^{2+} channels in vascular smooth

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muscle and cardiac muscle. DHPs, however, also inhibit Na^+ channels (Yatani *et al.*, 1988) and several types of K^+ channels: Kv (Avdonin *et al.*, 1997; Zhang *et al.*, 1997; Lin *et al.*, 2001), ATP-sensitive (Teramoto & Brading, 1998), and a number of other types of K^+ channels (Lin *et al.*, 1995; Sandle *et al.*, 1999). Large-conductance Ca^{2+}-activated K^+ channels are also modulated by DHPs (Fagni *et al.*, 1994; Klockner & Isenberg, 1989). We have shown that submicromolar concentrations of DHP Ca^{2+} channel antagonists, as well as the agonist BAY K 8644 with somewhat lower potency, can directly inhibit I_{to} in rabbit atrial myocytes (Gotoh *et al.*, 1991). The blockade of I_{to} by DHPs is characterized by a marked enhancement in its rate of inactivation, suggesting that DHPs block open I_{to} channels. Substantial block of I_{to} by 3 μM nifedipine has also been reported in rat ventricular myocytes (Jahnel *et al.*, 1994). Recently, it has been reported that even 30 μM nicardipine did not block the K^+ current consisting of the reconstituted human Kv4.3 channels (Calmels *et al.*, 2001). Previously, it has been reported that rat Kv4.2 was also much less sensitive to DHPs than rabbit I_{to} (Lin *et al.*, 2001).

The present study was undertaken to draw a conclusion for the confusing observations about susceptibility of reconstituted Kv4.3L, Kv4.2, and Kv1.4 channels to DHPs. We clearly show that nicardipine at submicromolar concentrations inhibited membrane currents through Kv4.3L, Kv1.4, and Kv4.2 channels expressed in HEK293 cells. DHPs are confirmed to be open-channel blockers of Kv4.3L, and have similar effects on Kv1.4 and Kv4.2.

Methods

Plasmid construction

Full-length Kv4.2 and Kv4.3L previously cloned from rat heart were ligated into the mammalian expression vector pcDNA3.1(+) (Invitrogen, CA, U.S.A.) (Ohya *et al.*, 1997). In addition, full-length Kv1.4 was cloned from rat heart by RT–PCR cloning using the following primers: (+): 5′-CCACGGATCCTTTGAACACCTACCCCCCAAAA-3′ corresponding to nucleotides −314 to 293 and (−): 5′-CCACGAATTCGCAATACACGTTTCGCCAAT-3′ corresponding to nucleotides 2087 – 2116 (GenBank accession number, X16002). The underlined sequences, *BamH*I (GGATCC) and *EcoR*I (GAATTC) recognition sites, were added to the PCR products to insert them into plasmid DNA in the proper orientation. Cloned cDNAs were sequenced by dideoxy sequencing methods using Thermo Sequenase Cycle Sequencing Kit (Shimadzu, Kyoto, Japan) with DSQ-2000.

Cell culture and transfection

Human embryonic kidney cell line (HEK293) was obtained from Health Science Research Resources Bank (HSRRB, Osaka, Japan) and maintained in minimum essential medium (Invitrogen, CA, U.S.A.) supplemented with 10% heat-inactivated fetal calf serum, penicillin (100 U/ml^{-1}), and streptomycin (100 μg ml^{-1}). Rat Kv1.4, Kv4.2, and Kv4.3L constructs described above were stably transfected into HEK293 cells using the calcium phosphate precipitation method, as previously described (Ohya *et al.*, 1997). In some experiments, rat K^+ channel interacting protein 2 (KChIP2) was coexpressed with Kv4.3L (Ohya *et al.*, 2001).

Electrophysiological methods

Transfected cells were dissociated and then seeded onto glass coverslips 1 – 2 days before use. Whole-cell membrane currents were recorded with the whole-cell voltage-clamp technique by using a CEZ-2400 amplifier (Nihon-Koden, Tokyo, Japan). All measurements were carried out at room temperature (23 ± 1°C). Membrane currents were recorded and stored as previously reported (Imaizumi *et al.*, 1990). The resistance of microelectrodes filled with internal solution was 2 – 4 MΩ. Series resistance compensation was set at 60%. For electrical recordings, a standard HEPES buffered solution of the following composition was used (mM): NaCl 137, KCl 5.9, $CaCl_2$ 2.2, $MgCl_2$ 1.2, glucose 14, and HEPES 10 (pH 7.4). The internal pipette solution contained (mM): KCl 140, $MgCl_2$ 4, ATP-2Na 5, EGTA 0.05, and HEPES 10 (pH 7.2).

The voltage dependence of I_{to} activation was measured using conventional double-pulse protocol as described previously (Hatano *et al.*, 2002). The membrane potential was held at −80 mV and depolarized to test potentials for 8 ms to activate I_{to} and then to −50 mV to measure the tail current. The tail current amplitude was normalized to the maximum in each cell and plotted against the test potentials. The data were fitted with a Boltzmann function and the voltage required for the half maximal activation and the slope factor were then determined. This double pulse was sequentially applied once every 10 s. The voltage dependence of I_{to} inactivation was also determined using a double-pulse protocol. I_{to} was activated and inactivated by depolarization from −80 mV to test potentials for 1 s, and then the remaining available channels were activated by the following depolarization to +20 mV. Normalization and the fitting of the data to Boltzmann function were performed. This double pulse was sequentially applied every 10 s. Data acquisition and analysis were carried out using the software (AQ and Cell Soft) developed in Dr Giles laboratory (University of Calgary, Calgary, Canada).

Data analysis

The dose – response curve for DHP-induced inhibition of I_{to} was determined by integrated currents and fitting a curve to each set of data based on the Hill equation:

$$F = 1/[1 + (K_d/[D]^n)]$$

where F is the fractional block ($F = 1 - I_{drug}/I_{control}$) at drug concentration [D], K_d is the concentration producing half-maximal inhibition, and n is the Hill coefficient. In Results, K_d was described as the concentration required for half inhibition, IC_{50}, since 'n' was reasonably close to 1.0.

Pooled data of fractional block were expressed as mean ± s.e.m. in the text and figures. Statistical significance between two and among multiple groups was determined by Student's t-test and Dunnett's test after one-way ANOVA, respectively. Significance was expressed in figures by $^*P < 0.05$ or $^{**}P < 0.01$.

Drugs

Dihydropyridine compounds (nicardipine and BAY K 8644) were obtained from Yamanouchi Pharmaceutical Co., Ltd (Tokyo, Japan), Sigma-Aldrich (Missouri, U.S.A.), and Funakoshi (Tokyo, Japan), which were dissolved in ethanol at a stock solution of 10 mM, and were protected from light during the entire experiments. Other chemicals were obtained from Wako Pure Chemical Industries (Osaka, Japan) and Dojindo (Kumamoto, Japan).

Results

Inhibition of Kv4.3L currents by nicardipine

Figure 1A,a shows typical families of macroscopic membrane currents carried by Kv4.3L channels expressed in HEK293 cells. In these experiments, each cell was depolarized to test potentials between −70 and +40 mV by 10 mV steps applied once every 10 s from the holding potential of −80 mV. The averaged peak outward current at +40 mV was 2549±381 pA ($n=11$). Outward currents recorded upon depolarization in native untransfected HEK293 cells exhibited slower activation, much smaller amplitude, and no inactivation (Figure 1A, a inset) (277±28 pA at +40 mV, $n=7$, $P<0.001$ vs Kv4.3L-expressing HEK). The cell capacitance was not changed by Kv4.3L expression (30.5±1.3 pF, $n=7$ and 25.7±2.2 pF, $n=11$ in native and Kv4.3L-expressing HEK, respectively; $p>0.05$). When cells were depolarized to +20 mV once every 20 s, the application of 1 μM nicardipine gradually reduced the amplitude of the peak outward current of Kv4.3L and this frequency-dependent effect reached the steady level within 3 min (Figure 1A, b).

A prominent effect of nicardipine was acceleration of the inactivation time course (see Figure 2c). An increase in concentration of nicardipine to 3 μM resulted in larger inhibition of Kv4.3L currents (Figure 1A, c). Figure 1B shows the current – voltage relations for the peak outward currents in the absence and presence of 1 or 3 μM nicardipine. The relative inhibition by nicardipine was not modulated by test potentials in the range of −20 and +40 mV (Figure 1C), suggesting that the inhibition of peak outward current by nicardipine is not voltage dependent ($P>0.05$ between test potentials each for 1

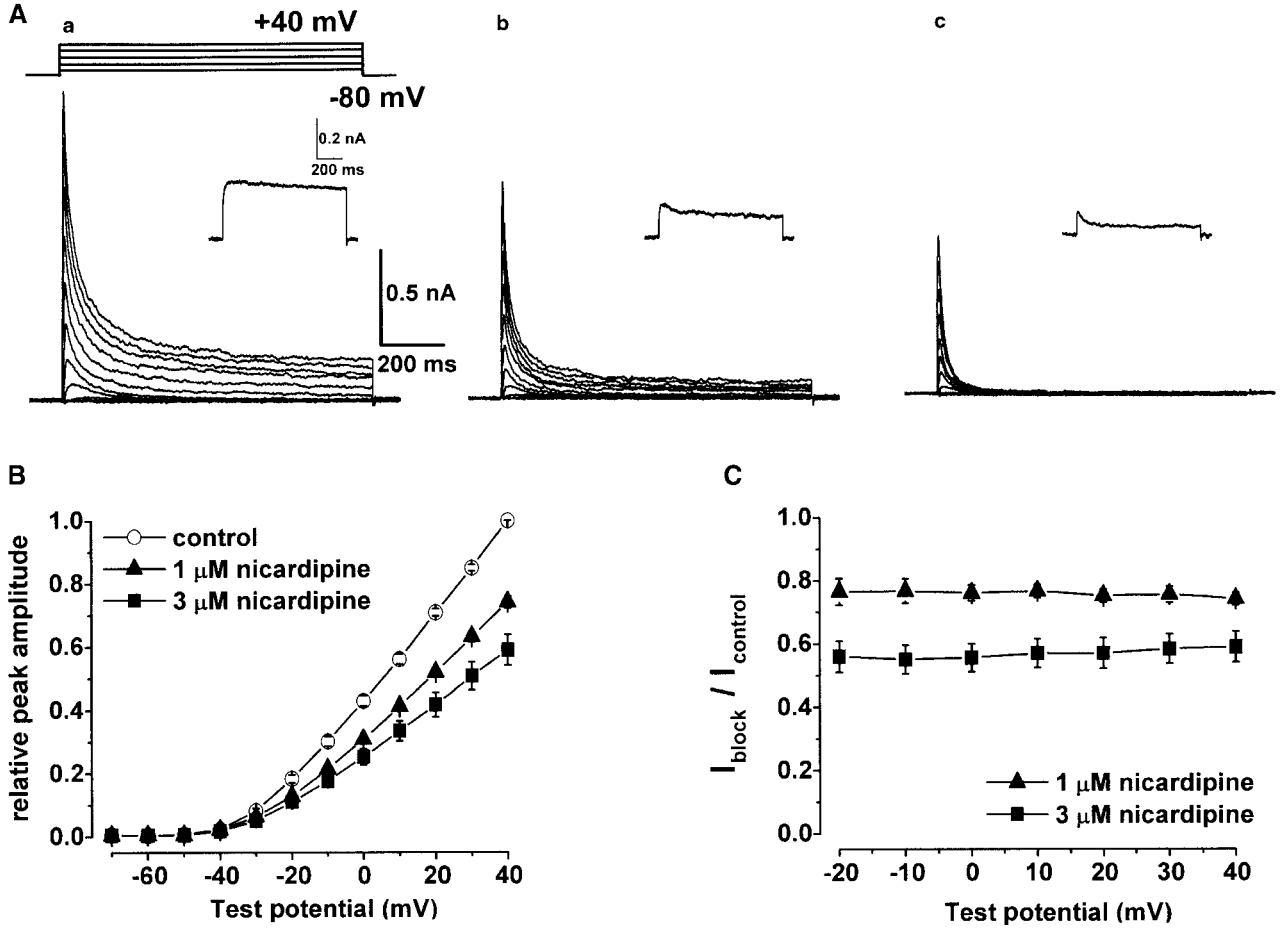

Figure 1 Effects of nicardipine on Kv4.3L currents in HEK293 cells. (A) Each HEK293 cell expressing Kv4.3L was depolarized for 1 s from −80 mV in 10 mV steps, once every 10 s under whole-cell voltage clamp. Whole-cell K^+ currents were recorded in the absence (a) and the presence of 1 (b) and 3 μM (c) nicardipine. Insets show K^+ currents elicited by depolarization from − 80 to +20 mV in a native HEK293 cell in the absence (a) and presence of 1 (b) and 3 μM nicardipine (c). (B) Current – voltage relations in the absence (○ $n=11$) and the presence of 1 ($n=7$) and 3 μM ($n=5$) nicardipine. In each cell, the peak amplitude was normalized by that at +40 mV in the absence of nicardipine. Data are expressed as means±s.e.m. (vertical bars). (C) Voltage dependence of the inhibition by 1 and 3 μM nicardipine. The peak amplitude of currents in the presence of nicardipine (I_{block}) was normalized to that at each voltage in the absence ($I_{control}$).

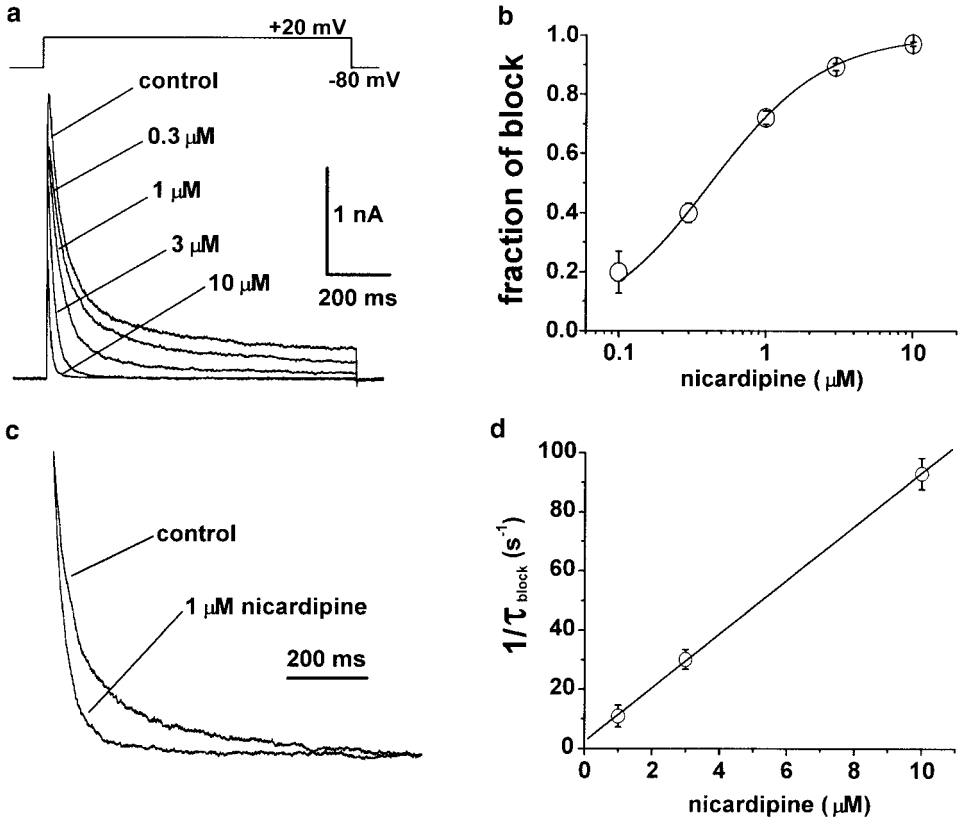

Figure 2 Inhibition of Kv4.3L currents and acceleration of their decay time course by nicardipine. (a) Current traces recorded at +20 mV in the absence and the presence of 0.3, 1, 3, and 10 μM nicardipine are superimposed. (b) Dose – response relations for nicardipine-induced inhibition of integrated Kv4.3L currents. Summarized data were obtained from experiments as shown in (a). Integrated values of currents at a selected concentration of nicardipine were normalized to integrated values of current under control condition. Mean values ± s.e.m. (as vertical bars) are plotted against the concentrations of nicardipine for $n = 4 - 9$ cells. The solid line denotes a fitted curve using the Hill equation with an IC_{50} and Hill coefficient of 0.42 μM and 1.12, respectively. (c) Kv4.3L currents were activated by depolarization from −80 to +20 mV. Recordings in the absence and the presence of 1 μM nicardipine were superimposed after adjusting their peak amplitudes. The inactivation time course of Kv4.3L currents in the absence and the presence of nicardipine was well fitted with a double-exponential function (control: $n = 11$; 1 μM nicardipine: $n = 10$). (d) Rate of open-channel block ($1/\tau_{block}$) as a function of the concentration. The calculated $1/\tau_{block}$ from the data in Table 1 was plotted against nicardipine concentration. The apparent rate constants of binding and unbinding (k_{+1} and k_{-1}) for the nicardipine were obtained for the slope and intercept of the best-fit line, according to the equation, $1/\tau_{block} = k_{+1}[D] + k_{-1}$.

and 3 μM nicardipine). Delayed rectifier-type outward currents upon depolarization in native HEK were also blocked by nicardipine (Figure 1A, b and c, insets); the amplitude of sustained outward current at +20 mV was reduced to less than 10% by 3 μM nicardipine ($n = 3$).

Kv4.3L currents recorded at +20 mV were reduced by nicardipine in a concentration-dependent manner as shown in Figure 2a. To evaluate this block semiquantitatively, the current at +20 mV was integrated and the relative decrease in integrated current by nicardipine vs the control value was expressed as fractional block in the same way used in the previous study (Gotoh *et al.*, 1991). Each set of data was fitted by the Hill equation (see Methods). These results are summarized in Figure 2b by superimposing a curve-fitted relation based on averaged IC_{50} value of 0.42 ± 0.05 μM ($n = 4 - 9$ at each concentration) and the Hill coefficient of 1.12 ± 0.11 ($n = 9$). When relations between fractional block and concentrations of nicardipine were evaluated from the inhibition of peak current and the current at the end of 1000 ms pulse, the IC_{50}'s were 4.63 ± 1.82 ($n = 9$) and 0.30 ± 0.06 μM ($n = 9$), respectively. Hill coefficients were 0.70 ± 0.11 and 1.14 ± 0.15, respectively. The racemic mixture of the DHP Ca^{2+} agonist BAY K 8644 also reduced Kv4.3L currents. The IC_{50} and Hill coefficient of BAY K 8644 were 1.74 ± 0.11 μM and 1.19 ± 0.24 ($n = 4$), respectively, when the inhibition of integrated currents was evaluated. These results suggest that DHPs bind a single binding site in the Kv4.3L channel.

Nicardipine facilitated the inactivation of Kv4.3L currents

As shown in Figure 2c in which the peak values of the control and blocked Kv4.3L records are set equal, a very pronounced feature of the inhibition of Kv4.3L currents by nicardipine was the increase in the apparent rate of current inactivation during depolarization. The inactivation time course of Kv4.3L currents in the absence of a drug was well fitted by the sum of two exponential components:

$$I(t) = A_{fast} \exp(-t/\tau_{fast}) + A_{slow} \exp(-t/\tau_{slow}) + A_0$$

where A_{fast} and τ_{fast} are the initial amplitude and time constant of the fast component of inactivation, respectively. A_{slow} and τ_{slow} are those for the slow component. A_0 is a time-independent component. In the absence of a drug, the τ_{fast} at +20 mV was 44.4 ± 2.6 ms, which was much smaller than the

Table 1 Concentration dependent effects of nicardipine on parameters of Kv4.3L current inactivation

Nicardipine (μM)	*Inactivation rate*			
	τ_{fast} (ms)	τ_{slow} (ms)	$A_{fast}/(A_{fast}+A_{slow})$ (%)	n
Control	44.4±2.6	290.2±22.5	76.8±1.4	11
0.1	43.2±3.6	260.6±23.2	76.2±2.6	7
0.3	41.0±3.0	232.5±23.9	80.2±1.0	10
1	32.0±2.9**	166.7±18.7**	88.7±1.3**	10
3	19.7±1.5**	142.5±45.1**	95.6±1.0**	8
10	8.7±0.5**	38.2±3.4**	97.0±1.0**	4

**$P<0.01$ vs control.

τ_{slow}, 290±23 ms ($n=11$) (Table 1). The fast component of the inactivation current was predominant at +20 mV, with a relative contribution of 77% (determined as $A_{fast}/(A_{fast}+A_{slow})$). Thus, the inactivation rate of Kv4.3L current was mainly determined by τ_{fast} under control conditions. Even after application of 1 μM nicardipine, the current decay was fitted with a double-exponential function and both time constants were decreased significantly. The contribution of fast component was significantly increased by nicardipine at 1 μM to 89% (Table 1). As previously described (Slawsky & Castle, 1994; Sanchez-Chapula, 1999), we calculated the time constant of nicardipine block (τ_{block}) with an approximation of the channel-blocking kinetics by nicardipine using the following equation:

$$1/\tau_{decay} = 1/\tau_{block} + 1/\tau_{inactivation}$$

The τ_{fast} in the absence of nicardipine approximated $\tau_{inactivation}$ as described above. The decay of Kv4.3L currents in the presence of 1, 3, or 10 μM nicardipine included both the inactivation *per se* and the block of currents by nicardipine. The τ_{fast} in the presence of nicardipine approximated τ_{decay}. Then, we plotted $1/\tau_{block}$ against nicardipine concentration (Figure 2d). The data were fitted by a line with a least-squares linear fit:

$$1/\tau_{block} = k_{+1}[D] + k_{-1}$$

where k_{+1}, and k_{-1} are the apparent rate constants of binding and unbinding for the drug, respectively. The slope and intercept for the fitted equation yield $k_{+1}=9.1\times10^6\,M^{-1}\,s^{-1}$ and $k_{-1}=2.4\,s^{-1}$. From these data, the calculated apparent K_d (k_{-1}/k_{+1}) was 0.26 μM, which is in good agreement with the IC_{50} value of 0.42 μM obtained from the concentration – response curve. These suggest that the binding of the nicardipine with Kv4.3L channel is independent of the inactivation process.

Changes in recovery from inactivation of Kv4.3L currents by nicardipine

Effects of 1 μM nicardipine on the voltage dependence of Kv4.3L current activation and steady-state inactivation were examined using conventional double-pulse protocols, respectively (see Methods). Figure 3a shows the summarized data for the activation in the absence and presence of 1 μM nicardipine. The half-activation voltage ($V_{1/2}$) was obtained by fitting of Boltzmann equation to each set of data. $V_{1/2}$ was slightly shifted to negative potentials by 1 μM nicardipine (control: $V_{1/2}=-10.3\pm0.7$ mV; 1 μM nicardipine: $V_{1/2}=-14.1\pm1.4$ mV, $P<0.05$ vs control; $n=5$ for each). Figure 3b shows the summarized data for the inactivation in the absence and presence of 1 μM nicardipine. $V_{1/2}$ was slightly shifted to a negative potential by 1 μM nicardipine (control: $V_{1/2}=-53.1\pm0.9$ mV; 1 μM nicardipine: $V_{1/2}=-58.6\pm1.7$ mV, $P<0.05$ vs control; $n=6$ for each).

The time course of recovery from inactivation of Kv4.3L was studied using conventional paired-pulse protocol (Figure 3c, d). The peak amplitude of Kv4.3L currents elicited by the second pulse was normalized with that elicited by the first pulse, and plotted against the interpulse duration (Δt). The recovery time course of Kv4.3L currents was well fitted by a single-exponential function (Figure 3c, d). The time constant (τ) in the presence of 1 and 3 μM nicardipine at −80 mV was 336.3±40.7 ms ($n=6$, $P<0.05$ vs control) and 516.3±32.9 ms ($n=4$, $P<0.01$ vs control), and both values were significantly larger than that in the absence (control: 230.9±18.3 ms, $n=9$) (Figure 3c, d).

Use and frequency dependence of nicardipine-induced block of Kv4.3L currents

Figure 4 shows the results obtained from experiments to investigate the use and frequency-dependent block of Kv4.3L currents by 1 μM nicardipine. In Figure 4a, a cell was activated by a set of 10 depolarizing clamp pulses and the control current was recorded at 'a'. Just after the cessation of the stimulation, 1 μM nicardipine was applied. A second set of clamp pulses was started 7 min after the application of nicardipine. The 10 sequential recordings obtained at 'b' are shown in the right panel. The block of the peak current at the first pulse in the second set was identical to that at the tenth (0.78±0.05 and 0.74±0.03, $n=4$, $P>0.05$). These results suggest that a tonic block developed in the period during which no channel opened and that practically no use dependence can be observed in these conditions (at 0.1 Hz).

Frequency dependence of the block was examined in Figure 4b. In the absence of nicardipine, 10 depolarizing pulses of 200 ms in duration and 100 mV amplitude (from −80 to +20 mV) were applied at 0.1, 0.5, and 1 Hz, respectively, with an interval of 1 min between each set of pulses. After the treatment with 1 μM nicardipine for 6 min, the same protocol was repeated. The peak current amplitude elicited by the first clamp step at each frequency in the absence of nicardipine was defined as unity. In the absence of nicardipine, the peak amplitude of I_{to} by the second pulse was not changed at 0.1 and 0.5 Hz. However, it was significantly reduced at 1 Hz, suggesting a time-dependent increase in inactivation. In the presence of nicardipine, the block showed no progressive change at 0.1 Hz, tended to increase at 0.5 Hz ($P>0.05$ between first and tenth pulses) and significantly increased at 1 Hz ($P<0.05$). To evaluate the use and frequency dependency of nicardipine-induced block more clearly, the influence of

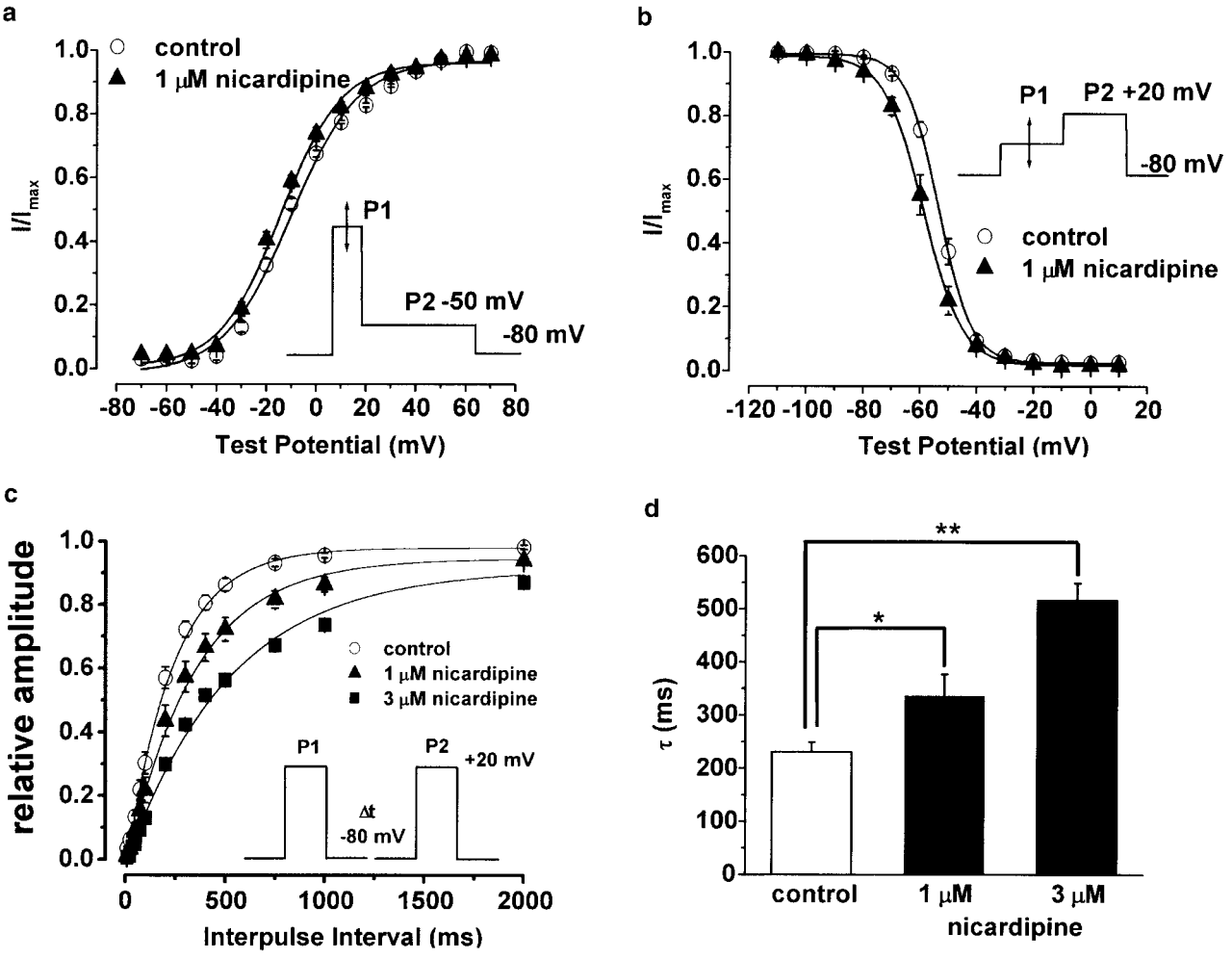

Figure 3 Effects of nicardipine on the voltage dependence of activation and steady-state inactivation, and the time course of recovery from inactivation of Kv4.3L currents. (a) Activation curves in the absence (○) and the presence of 1 μM nicardipine. The tail-current amplitude was normalized to the maximum in each cell, and plotted against the test potentials. These data were fitted with the Boltzmann function and the voltage required for the half-maximal activation and the slope factor were determined ($n=5$ for each). (b) Steady-state inactivation curves in the absence and the presence of 1 μM nicardipine. Current normalization and the fitting of these data with the Boltzmann function were performed in a similar manner as those for the activation ($n=6$ for each). (c) The time course of recovery from inactivation at -80 mV in the absence (○) and presence of 1 or 3 μM nicardipine. Summarized data were plotted as the relative amplitude of I_{to} (I_{P2}/I_{P1}) against the interpulse interval (Δt) (ms). The recovery time course was best described by a single-exponential function (control, $n=9$; 1 μM nicardipine, $n=6$; 3 μM nicardipine, $n=4$) and summarized τs are shown in (d).

accumulated inactivation at 1 Hz in the absence of nicardipine was determined as follows; the peak amplitude elicited by each pulse in the presence of nicardipine was divided by the corresponding one in the absence and replotted in Figure 4c. The use-dependent increase in the block was observed only at 1 Hz. The relative amplitudes at the tenth pulse were 0.77 ± 0.08, 0.67 ± 0.04, and 0.58 ± 0.02 ($P<0.05$ between 0.1 vs 1 Hz), indicating the frequency-dependent block of Kv4.3L by 1 μM nicardipine.

Coexpression of KChIP2 with Kv4.3L and effects of nicardipine

Coexpression of KChIP2 with Kv4.3L (Kv4.3L/KChIP2S) markedly increased the current density (259 ± 26 pA pF^{-1}, $n=6$, $P<0.01$ vs Kv4.3L alone: 101 ± 14 pA pF^{-1}, $n=11$) and changed both the activation and inactivation kinetics (Figure 5a), as has been reported in the combination of Kv4.3 and KChIP2 (Bahring *et al.*, 2001; Decher *et al.*, 2001; Patel *et al.*, 2002). Cell capacitance was not changed by this coexpression (21.1 ± 1.0 pF, $n=6$, $P>0.05$ vs Kv4.3L alone: 25.7 ± 2.2 pF, $n=11$). The inactivation phase of Kv4.3L/KChIP2S at +20 mV was well described by the sum of the two exponential functions and the parameters are as follows: τ_{fast} 46.4 ± 4.6 ms ($P>0.05$ vs Kv4.3L alone), τ_{slow}: 245.8 ± 67.3 ($P>0.05$ vs Kv4.3L alone). The contribution of fast inactivation component, $A_{fast}/(A_{fast}+A_{slow})$, was significantly increased by the co-expression (0.93 ± 0.03, $n=6$, $P<0.01$ vs Kv4.3L alone) and this resulted in a crossover of the inactivating phases of control Kv4.3L currents and Kv4.3L/KChIP2S. The peak amplitude and the inactivation rate of Kv4.3L/KChIP2S were reduced and accelerated, respectively, by application of nicardipine. These effects were concentration-dependent (Figure 5b). The apparent IC_{50} value and Hill coefficient of nicardipine were 0.77 ± 0.14 μM and 0.75 ± 0.08 ($n=5$), respectively (Figure 5c). The value of τ_{fast} was significantly

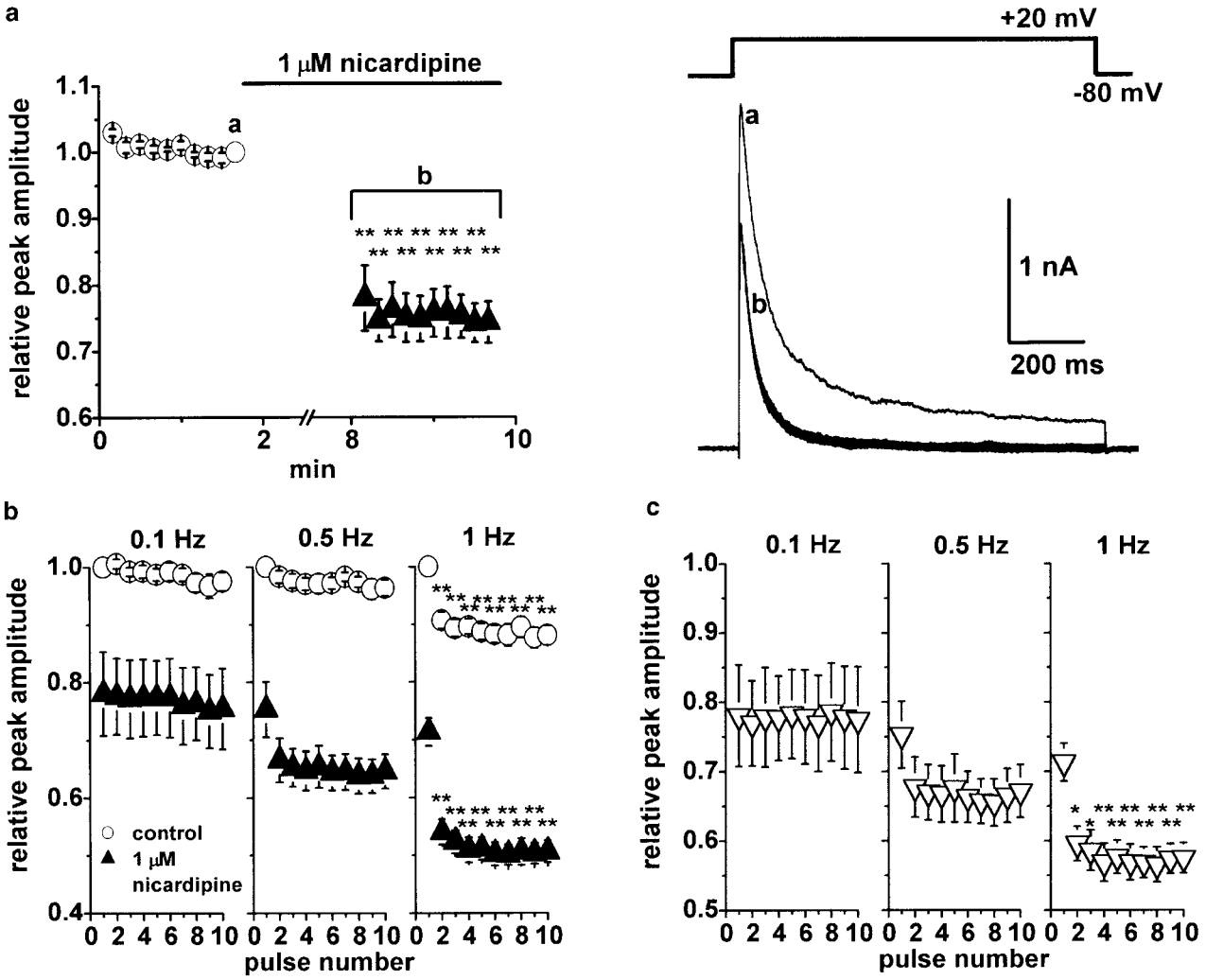

Figure 4 Use- and frequency-dependent effect of nicardipine on Kv4.3L currents. (a) Two sets of 10 rectangular command pulses depolarized a cell from −80 to +20 mV for 1 s at 0.1 Hz and 7 min elapsed between these two tests. Nicardipine (1 μM) was applied just after the first train of voltage-clamp depolarization (left panel). The peak amplitude of Kv4.3L current elicited by the 10th pulse in the first set was taken as the control (1.0) in ordinate. The Kv4.3L currents recorded at the times indicated by 'a' and 'b' in the pulse train are shown in the right panel. These experiments were tested in four cells. Changes in I_{to}, which were statistically significant, are indicated by **($P<0.01$). (b) Frequency dependence of nicardipine-induced block was examined in the range of 0.1, 0.5, and 1 Hz. In the absence of nicardipine (○), 10 depolarizing pulses of 200 ms in duration and 100 mV in amplitude (from −80 to +20 mV) were applied at 0.1, 0.5, and 1 Hz, respectively, with an interval of 1 min between each set of pulses. After the treatment with 1 μM nicardipine for 6 min, the same protocol was repeated. The peak amplitude elicited by the first pulse at each frequency in the absence of nicardipine was taken as unity. (c) The peak amplitude elicited by each pulse in the presence of nicardipine in 'b' was divided by the corresponding one in the absence and replotted against time (▽). Number of experiments was four to five. Statistically significant effects assessed vs the first pulse in each group were indicated by *$P<0.05$ and **$P<0.01$).

reduced by nicardipine at a concentration of 1 μM and above (Figure 5d). Similar to Figure 2d, we plotted $1/\tau_{block}$ (approximated by $1/\tau_{fast}$) against nicardipine concentration, and the data were fitted by a line with a least-squares linear fit (not shown). The slope and intercept for the fitted equation yield $k_{+1}=7.0\times10^6\,M^{-1}\,s^{-1}$ and $k_{-1}=5.6\,s^{-1}$. From these data, the calculated apparent K_d (k_{-1}/k_{+1}) was 0.80 μM, which is again in good agreement with the IC_{50} value of 0.77 μM obtained from the concentration – response curve.

Effects of nicardipine on other K^+ channel subtypes which can generate I_{to}

We have also examined the effects of nicardipine on Kv4.2 and Kv1.4 currents in HEK293 cells. In these experiments, currents owing to Kv4.2 and Kv1.4 channels were activated by depolarization from −80 to test potentials between −50 and +40 mV. As expected, these records exhibited the typical features of transient outward currents. These K^+ currents activated at +20 mV were recorded in the absence and presence of nicardipine at five different concentrations in a range of 0.1 and 10 μM (Figure 6A, a and B, a). The concentration dependence of the inhibitory effects of nicardipine was quantified in the same manner as described in Figure 2. The resultant IC_{50} values of nicardipine were 0.62±0.14 and 0.80±0.21 μM for Kv4.2 and Kv1.4, respectively ($n=7$ and 5, respectively) (Figure 6A, b and B, b), and the Hill coefficients were 0.99±0.10 and 1.11±0.10, respectively. Moreover, application of nicardipine accelerated the inactivation rate in both Kv4.2 and Kv1.4 currents in a

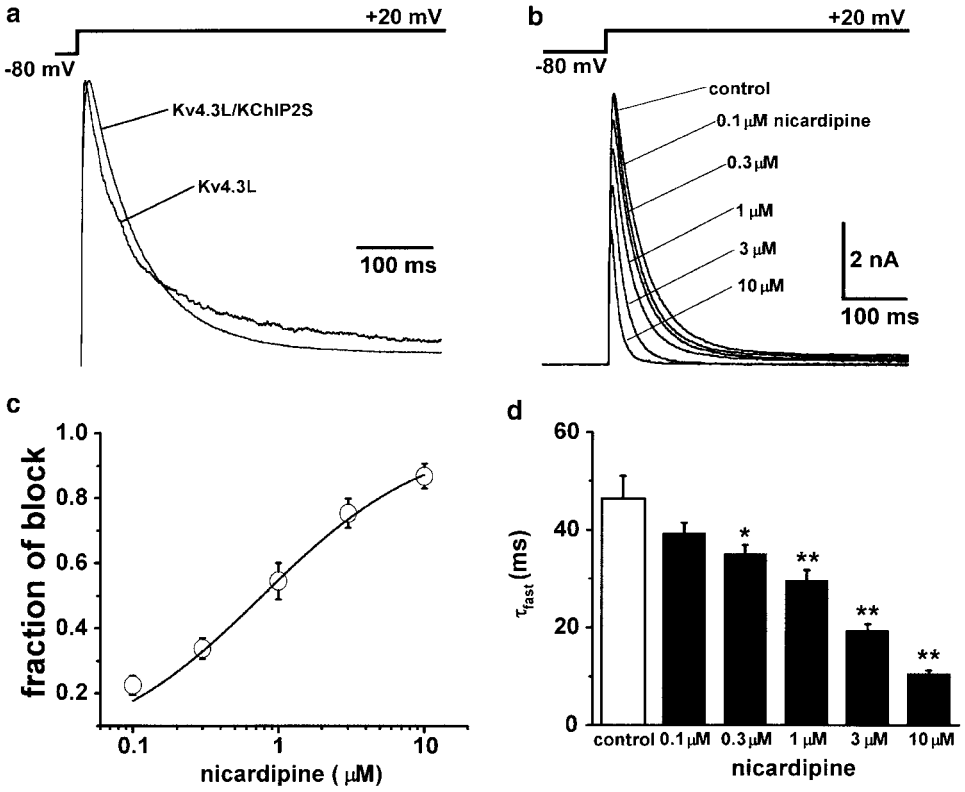

Figure 5 Effects of coexpression of KChIP2S with Kv4.3L on nicaridipine-induced block. (a) Currents were elicited by depolarization from −80 to +20 mV in the absence of nicardipine in HEK293 cells expressing Kv4.3L alone and coexpressing Kv4.3L and KChIP2S. The peak amplitudes of two traces were made equal on the computer. (b) Effects of nicardipine on Kv4.3L/KChIP2S currents were examined by cumulative addition of nicardipine in the range of 0.1 – 10 μM. (c) The solid line denotes a curve fit obtained using the Hill equation, IC_{50} (0.77 μM) and Hill coefficient (0.75). The fractional block was obtained by dividing the value of integrated current in the presence of nicardipine by that in the absence of nicardipine using data obtained from three to four separate experiments. (d) The inactivation time course of Kv4.3L/KChIP2S currents was well fitted by the sum of two exponential functions. The acceleration of the inactivation rate of I_{to} by nicardipine was evaluated by changes in the time constant of the fast component (τ_{fast}). Statistical significance vs control is indicated by $^*P<0.05$ and $^{**}P<0.01$.

manner very similar to this effect on Kv4.3L. The time course of inactivation of Kv4.2 and Kv1.4 were well described by the sum of the two exponential functions. The acceleration of inactivation by nicardipine was evaluated by changes in the time constant of fast component of inactivation, since $A_{fast}/(A_{fast}+A_{slow})$ was over 0.8 in both Kv4.2 and Kv1.4 (0.87 ± 0.02, $n=7$ and 0.91 ± 0.02, $n=10$, respectively). τ_{fast}s of both Kv4.2 and Kv1.4 were reduced in concentration-dependent manners (Figure 6A, c and B, c). Further details of nicardipine-induced block of Kv4.2 and Kv1.4 were not examined.

Discussion

The present study shows that DHP compounds (both DHP Ca^{2+} antagonists and agonists) exhibit potent block of rat Kv4.3L, Kv4.2, and Kv1.4 K^+ channels, which were heterologously expressed in HEK293 cells. These results provide direct evidence for the mechanism of DHP action at submicromolar concentrations on the Ca^{2+}-independent transient outward current, I_{to} in cardiac myocytes (Gotoh *et al.*, 1991) and I_A in bovine adrenal zona fasciculata cells (Mlinar & Enyeart, 1994).

DHPs inhibit several types of K^+ channels in addition to L-type voltage-dependent Ca^{2+} channels (Gotoh *et al.*, 1991; Valmier *et al.*, 1991; Fagni *et al.*, 1994; Jahnel *et al.*, 1994; Mlinar & Enyeart, 1994; Lin *et al.*, 1995; Zhang *et al.*, 1997; Teramoto & Brading, 1998; Sandle *et al.*, 1999; Lin *et al.*, 2001). The inhibitory effects of nifedipine on cloned Kv1.1, Kv1.2, Kv1.3, Kv1.5, and Kv3.1 have been systematically examined by Grissmer *et al.* (1994). In most of these reports, however, the concentration of DHPs required for the block of K^+ current is much higher than that for therapeutic doses ($>10\,\mu$M). In contrast, I_{to} in rabbit atrial myocytes can be blocked by DHPs at submicromolar (Gotoh *et al.*, 1991) concentrations. The IC_{50}s of nicardipine and Bay K8644 for the block of I_{to} were 0.63 and 7 μM, respectively, when the inhibition of integrated I_{to} was evaluated. I_A in bovine adrenal zona fasciculata cells (Mlinar & Enyeart, 1994) and delayed rectifier K^+ channels in human colonic crypts (Sandle *et al.*, 1999) are also blocked by DHPs at low concentrations.

Based on our previous findings that DHPs effectively block I_{to} in rabbit atrial myocytes (Gotoh *et al.*, 1991) and our preliminary observations obtained by qualitative PCR analyses that transcripts responsible for I_{to} in rabbit atrial myocytes are mainly Kv4.3L, Kv4.2, and Kv1.4 (Ohya, unpublished observation), we expect that, at least, one of these K^+ channel α subunits would be a target for DHPs. Recently, however, Calmels *et al.* (2001) have reported that verapamil inhibited hKv4.3 currents and accelerated current inactivation, but nicardipine did not affect hKv4.3 currents at

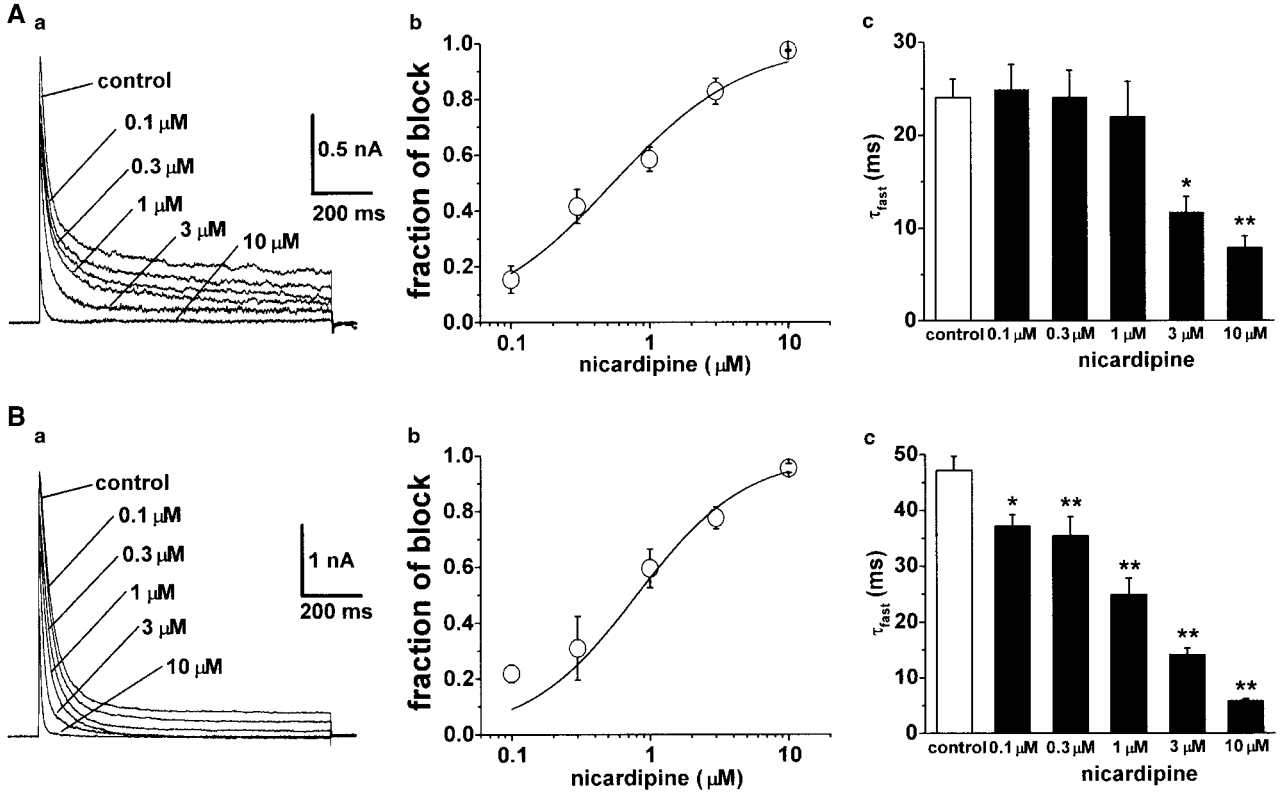

Figure 6 Effects of nicardipine on Kv4.2 and Kv1.4 currents. The Kv4.2 (A, a) and Kv1.4 (B, a) current traces recorded at +20 mV in the absence and the presence of 0.1, 0.3, 1, 3, and 10 μM nicardipine were superimposed. Concentration – response relations for nicardipine-induced inhibition of integrated currents of Kv4.2 and Kv1.4 are shown in (A,b) and (B,b) respectively. The values of integrated currents in the presence of nicardipine were normalized to the data obtained under control condition. Numbers of cells examined were four to seven and three to five for Kv4.2 and Kv1.4, respectively. The solid lines denote curve fits obtained using the Hill equation, IC_{50}'s (0.62 and 0.80 μM, respectively) and Hill coefficients (0.99 and 1.11, respectively). The inactivation time course of Kv4.2 and Kv1.4 currents in the absence was well fitted by the sum of two exponential functions. The acceleration of the inactivation time course of Kv4.2 and Kv1.4 currents by nicardipine was evaluated by changes in the time constant of fast component (τ_{fast}) in (A, c) and (B, c), respectively. Statistical significance vs control was indicated by $^*P<0.05$ and $^{**}P<0.01$.

concentrations up to 30 μM in HEK293 cell expression system (Calmels *et al.*, 2001). In addition, Lin *et al.* (2001) found that nifedipine blocked rat Kv4.2 currents at only high concentrations and the IC_{50} value in the integrated currents was 29 μM in HEK293 expression system (Lin *et al.*, 2001). These findings suggested that DHPs might not block I_{to} (or I_A) and currents due to cloned Kv4.2 and Kv4.3. In contrast, our results show that nicardipine can block rat Kv4.3L currents in a dose-dependent manner; the IC_{50} values were 4.3 and 0.42 μM, when the reduction of peak current and integrated current was determined, respectively. A similar pattern of block of rat Kv1.4 and Kv4.2 channel currents by nicardipine was observed, and IC_{50}'s for integrated currents were 0.80 and 0.62 μM, respectively. The reason why the present results are markedly different from those in previous two studies is not clear, but could be because of the conditions for expression and/or cell culture.

Our study was, however, performed using the same expression system, HEK293, as the previous study of hKv4.3L. The total number of amino acids of human and rat Kv4.3L is identical, 655. In fact, only filve amino acids are different between human and rat Kv4.3L; D152 in hKv4.3L is E152 in rKv4.3L in the N-terminal domain, G239 is V239 in S2 domain, L375 is P375 in pore domain, P540 is S540 and V654 is A654 in the C-terminal domain, respectively. It remains an interesting possibility that one or more of the five amino acids are responsible for the marked difference in susceptibility to DHPs in rat and human Kv4.3L. This is, however, rather unlikely, since our results obtained using rat Kv4.2 in this study are also markedly different from those by Lin *et al.* (2001). The amino-acid sequences of Kv4.3, Kv4.2, and Kv1.4 in rabbit heart are not identified; therefore we used clones from the rat for the expression experiments in this study. It has been reported that the inactivation rate of I_{to} in rat ventricular myocytes is markedly accelerated by nifedipine at concentrations $\geqslant 1$ μM (Jahnel *et al.*, 1994). We have confirmed these results in rat ventricular myocytes (Hatano, unpublished observation). These findings are consistent with the high susceptibility of rat cloned Kv4.3, Kv4.2 and Kv1.4 channels to DHPs and also with the IC_{50} of nicardipine in rabbit atrial myocytes (0.63 μM).

The features of nicardipine-induced block of Kv4.3L in HEK cells were consistent with those reported for the block of I_{to} in rabbit atrial myocytes. Some antiarrhythmic drugs, such as quinidine, tedisamil, disopyramide, propafenone, and flecainide, inhibit I_{to} in a fashion that suggests an open-channel block mechanism (Slawsky & Castle, 1994; Yamashita *et al.*, 1995; Wettwer *et al.*, 1998; Sanchez-Chapula, 1999). Thus, the inhibition of I_{to} by these agents and DHPs is characterized by a concentration-dependent reduction in peak

amplitude and, more prominently, by an acceleration of the inactivation rate. However, some differences have been identified between the properties of these agents. For example, the blocking action of quinidine on Kv4.3L currents is dependent on the test potential; however, the blocking action of nicardipine was independent of the test potential as well as tedisamil, disopyramide, and flecainide. In addition, unlike propafenone (Duan *et al.*, 1993), nicardipine, flecainide, quinidine, and disopyramide all produced significant changes in the time course of I_{to}/Kv4.3L current recovery from inactivation. This phenomenon may explain the increase in the inhibition of Kv4.3L current by nicardipine at higher frequencies of depolarization pulses. Indeed, nicardipine produced a small amount of use-dependent block of Kv4.3L current only at higher frequencies of stimulation.

Another characteristic of nicardipine-induced block of Kv4.3L was a prominent tonic effect, which developed without channel activation by stimulation. Although this could be because of the binding of nicardipine to channels in the resting state, the remaining current elicited by depolarization after the tonic block developed, showed accelerated inactivation rate. This pattern of effect is different from that in the presence of 4-aminopyridine, an agent that has the highest affinity to resting Kv4 channels (Tseng *et al.*, 1996). Since DHPs have high lipophilicity, DHP-induced block may occur rapidly together with gating of channel-opening by approaching from the cytoplasmic site, as previously reported in cardiac Ca^{2+} channels by Kokubun & Reuter (1984). Alternatively, a small fraction of Kv4.3L channels could be inactivated at a holding potential of -80 mV and the binding of nicardipine to these inactivated channels could trap the channels in the state.

I_{to} plays a major role in the early phase of repolarization in cardiac myocytes. However, the issue whether the block in I_{to} by DHPs induces arrhythmogenic conditions remains unclear. Indeed, in rat ventricle, the block of I_{to} may result in significant prolongation of action potential duration (Pandit *et al.*, 2001). In addition, transgenic mice expressing a dominant-negative Kv4 subfamily exhibit a longer QT interval in the myocardium, but these mice do not develop spontaneous ventricular arrhythmias or tachyarrhythmias (Barry *et al.*, 1998). In contrast, it has been shown that marked reduction of Kv4 functional expression in KChIP2-deficient mice can be arrhythmogenic (Kuo *et al.*, 2001). A decrease in I_{Kur}, the ultrarapidly activating component of cardiac-delayed rectifier K^+ current, has been linked to arrhythmias, and I_{Kur} is associated with the Kv1 subfamily. However, nicardipine blocks Kv1 channels only at concentrations much higher than therapeutic doses (Grissmer *et al.*, 1994; Kv1.2, $IC_{50} = 18\,\mu$M; Kv1.5, $IC_{50} = 81\,\mu$M). These results suggest that the delayed rectifier channels in cardiac myocytes may not be clinical targets for DHPs. Moreover, neither fast nor slow components of cardiac-delayed rectifier K^+ currents (I_{Kr} and I_{Ks}) are susceptible to DHPs at therapeutic doses (Daleau *et al.*, 1997; Zhang *et al.*, 1999). It is noteworthy that $1\,\mu$M nicardipine reduced by 50% the peak Kv4.3L currents activated by 200 ms depolarization from -80 to $+20$ mV at 1 Hz. Therefore, I_{to}, in addition to I_{Ca}, can be one of the major targets of DHPs in cardiac myocytes, while volume-regulated Cl^- channel could be also another target (Tanaka *et al.*, 1997). The major effect of DHPs on cardiac action potential shape via block of I_{to} is supposed to be compensation of the decrease in plateau potential and subsequent action potential shortening via the inhibition of L-type Ca^{2+} channel currents (Gotoh *et al.*, 1991).

Recently, a number of important regulatory β subunits of Kv4 subfamily, such as KChIPs and NCS-1, have been identified in cardiac muscle (Nerbonne, 2001; Guo *et al.*, 2002). We have also identified that KChIP2S preferentially expressed in the heart (Ohya *et al.*, 2001). KChIPs specifically associate with the cytoplasmic N-termini of Kv4 subfamily, and the association of KChIPs results in an increase in current density, slower inactivation, and faster recovery from inactivation (An *et al.*, 2000; Patel *et al.*, 2002). Interestingly, our results showed that coexpression of KChIP2S did not essentially affect the inhibition of Kv4.3L by nicardipine. The IC_{50} of nicardipine was, however, slightly higher ($0.77\,\mu$M) in Kv4.3L/KChIP2S than Kv4.3L alone. This may be attributable to the fact that the rate of recovery of Kv4.3L is markedly increased by coexpression of KChIP2 (Bahring *et al.*, 2001; Decher *et al.*, 2001; Patel *et al.*, 2002). Since the combination of Kv4.3L and KChIP2 is the predominant K^+ channel responsible for I_{to} in human cardiac myocytes, the present results that the Kv4.3L currents activated by 200 ms depolarization from -80 to $+20$ mV at 1 Hz were half blocked by $1\,\mu$M nicardipine will be more important from a clinical point of view, if they are confirmed in Kv4.3L/KChIP2 at 37°C.

In summary, the present results, obtained by molecular pharmacological experiments with Kv4.3L, Kv4.2, and Kv1.4 stably expressed HEK293 cells, provide critical evidence supporting the high susceptibility of cardiac I_{to} to DHPs.

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