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Distinct properties of amlodipine and nicardipine block of the voltage-dependent Ca^{2+} channels $\text{Ca}_v1.2$ and $\text{Ca}_v2.1$ and the mutant channels $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive and $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive

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Abstract

The binding site within the L-type Ca^{2+} channel $\text{Ca}_v1.2$ for neutral dihydropyridines is well characterized. However, the contributions of the alkylamino side chains of charged dihydropyridines such as amlodipine and nicardipine to channel block are not clear. We tested the hypothesis that the distinct locations of the charged side chains on amlodipine and nicardipine would confer distinct properties of channel block by these two drugs. Using whole-cell voltage clamp, we investigated block of wild type $\text{Ca}_v2.1$, wild type $\text{Ca}_v1.2$, and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive, a mutant channel insensitive to neutral DHPs, by amlodipine and nicardipine. The potency of nicardipine and amlodipine for block of closed (stimulation frequency of 0.05 Hz) $\text{Ca}_v1.2$ channels was not different (IC_{50} values of 60 nM and 57 nM, respectively), but only nicardipine block was enhanced by increasing the stimulation frequency to 1 Hz. The frequency-dependent block of $\text{Ca}_v1.2$ by nicardipine is the result of a strong interaction of nicardipine with the inactivated state of $\text{Ca}_v1.2$. However, nicardipine block of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive was much more potent than block by amlodipine (IC_{50} values of 2.0 μM and 26 μM , respectively). A mutant $\text{Ca}_v2.1$ channel containing the neutral DHP binding site ($\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive) was more potently blocked by amlodipine ($\text{IC}_{50} = 41$ nM) and nicardipine ($\text{IC}_{50} = 175$ nM) than the parent $\text{Ca}_v2.1$ channel. These data suggest that the alkylamino group of nicardipine and amlodipine project into distinct regions of $\text{Ca}_v1.2$ such that the side chain of nicardipine, but not amlodipine, contributes to the potency of closed-channel block, and confers frequency-dependent block.

Keywords

Calcium channel blockers; Dihydropyridines; $\text{Ca}_v1.2$; Amlodipine; Nicardipine; Voltage Clamp

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1. Introduction

L-type voltage-gated Ca^{2+} channels are key modulators of contraction of vascular smooth and cardiac muscle, secretion of peptide hormones, and gene expression (Jones, 1998). The drugs that block L-type channels fall into three distinct chemical classes: dihydropyridines such as isradipine, amlodipine, and nicardipine; phenylalkylamines such as verapamil; and benzothiazepines such as diltiazem. Each of these classes of drugs bind the $\text{Ca}_v1.2 \alpha_1$ subunit (Hockerman et al., 1997a) (Striessnig et al., 1998) and block L-type channels in a characteristic manner (Lee and Tsien, 1983).

The binding determinants within transmembrane segments IIIS5, IIIS6, and IVS6 for dihydropyridines were identified using both biochemical and mutational approaches (Hockerman et al., 1997a; Mitterdorfer et al., 1998). A $\text{Ca}_v1.2$ channel with two mutations in transmembrane segment IIIS5 ($\text{Ca}_v1.2$ /dihydropyridine insensitive; Fig. 1A) was shown to be highly insensitive to neutral DHP drugs such as isradipine (Hockerman et al., 2000), nifedipine (Liu et al., 2003), and nisoldipine (Walsh et al., 2007). Conversely, several groups constructed high affinity binding sites for DHP drugs in normally insensitive $\text{Ca}_v2.3$ (Ito et al., 1997) or $\text{Ca}_v2.1$ (Sinnegger et al., 1997) (Hockerman et al., 1997b) channels (termed $\text{Ca}_v2.1$ /Dihydropyridine sensitive; Fig. 1B) by insertion of several L-type-specific amino acid residues.

The studies that led to the identification of the DHP binding site in L-type Ca^{2+} channels utilized high affinity, neutral drugs. The drug amlodipine (Arrowsmith et al., 1986) contains an ionizable alkylamino on the 2 position of the DHP ring (Fig. 1C). The drug nicardipine also contains an ionizable alkylamino group, but on the 5 position of the DHP ring (Fig. 1C) (Iwanami et al., 1979). The DHP pharmacophore is commonly described as a “flat boat” with the 4-aryl group in a pseudoaxial position forming the bowsprit of the boat and the DHP-ring nitrogen as the stern. Thus, the two non-equivalent sides of the molecule can be designated as “port” or “starboard”, based on orientation to the 4-aryl group (Goldman and Stoltefuss, 1991). In the crystal structure of the most active form of amlodipine, S-(−), the alkylamino substituent is on the port side of the molecule (Goldmann et al., 1992). In contrast, a solution NMR structure of the most active form of nicardipine, R-(+), placed the alkylamino group on the starboard side (Belciug and Ananthanarayanan, 1994). We hypothesized that the distinct positions of these charged groups in amlodipine and nicardipine could confer distinct channel blocking properties to these drugs. Therefore, we compared the ability of these two clinically relevant charged dihydropyridine drugs to block the L-type channel $\text{Ca}_v1.2$ (Snutch et al., 1991), the non-L-type channel $\text{Ca}_v2.1$ (Starr et al., 1991), and the mutant channels $\text{Ca}_v1.2$ /Dihydropyridine insensitive (Hockerman et al., 2000) and $\text{Ca}_v2.1$ /Dihydropyridine sensitive (Hockerman et al., 1997b). Our results suggest that nicardipine, but not amlodipine, accesses binding determinates outside of the canonical dihydropyridines site in mediating tonic and frequency-dependent block of the L-type Ca^{2+} channel $\text{Ca}_v1.2$.

2. Materials and Methods

2.1 Construction of Wild Type and Mutant Ca^{2+} Channels

The $\text{Ca}_v1.2$ (Snutch et al., 1991), $\text{Ca}_v2.1$ (Starr et al., 1991), and $\text{Ca}_v1.2$ /Dihydropyridine insensitive (Hockerman et al., 2000) (Hockerman et al., 2000) channels were sub-cloned into the pCDNA3 expression vector (Invitrogen, Carlsbad, CA). The DHP sensitive $\text{Ca}_v2.1$ mutant, $\text{Ca}_v2.1$ /Dihydropyridine sensitive (Hockerman et al., 1997b), was in the expression vector pMT-2 (Genetics Institute, Boston, MA). The desired mutations were verified, and the integrity of the clones was confirmed by cDNA sequencing and extensive restriction digest analysis.

2.2 Cell Culture

Human tsA-201 cells, a simian virus 40 (SV40) T-antigen expressing derivative of the human embryonic kidney cell line HEK293, were maintained in monolayer culture in DMEM/F-12 (GIBCO BRL/Life Technologies, Grand Island, NY) enriched with 10% fetal bovine serum (Hyclone, Logan, UT) and incubated at 37° C in 10% CO₂.

2.3 Expression of Ca²⁺ Channels

tsA-201 cells were co-transfected with wild type and mutant Ca_v1.2 subunits, β_{1b} (Pragnell et al., 1991), $\alpha_2\delta$ (Ellis et al., 1988), and enhanced green fluorescent protein (GFP) (Clontech, Palo Alto, CA) such that the molar ratio of the plasmids was 1:1:1:0.8. Cells were transfected using the GenePorter reagent (Gene Therapy Systems, San Diego, CA) and cells were re-plated at low density 20–24 h after transfection. Experiments were conducted 20–48 h after re-plating.

2.4 Electrophysiology

Transfected cells were recognized by GFP fluorescence at 510 nM with excitation at 480 nM. Barium currents through Ca²⁺ channels were recorded using the whole-cell configuration of the patch-clamp technique. Patch electrodes were pulled from VWR micropipettes (VWR, West Chester, PA) and fire-polished to produce a resistance of 2–3 M Ω . Currents were recorded using a List EPC7 patch-clamp amplifier and filtered at 1 or 2 kHz (8-pole Bessel filter, -3 dB; LPF 8, Warner Instruments, Hamden, CT). Voltage pulses were applied and data were acquired using pClamp8 software (Axon Instruments, Foster City, CA). Voltage-dependent leak currents were subtracted using an on-line P/–4 subtraction paradigm. Drugs were prepared as 10 mM stock solutions in 70% ethanol/30% water, dissolved in bath saline, and applied to cells using a cf-8VS perfusion system (Cell MicroControls, Norfolk, VA) with constant exchange of the bath solution. Barium current was measured in a bath saline containing (in mM) Tris (150), MgCl₂ (2) and BaCl₂ (10). The intracellular saline contained N-methyl-D-glucamine (130), EGTA (10), HEPES (60), MgATP (2) and MgCl₂ (1). The pH of both solutions was adjusted to 7.3 with methanesulfonic acid. Nicardipine (Sigma, St. Louis, MO) and amlodipine (gift of Pfizer, Sandwich, England) were diluted to the indicated concentrations in extracellular solution. Since the resolved enantiomers of amlodipine and nicardipine are not readily available, we used the racemates in this study. The S-(–) isomer of amlodipine is ~1000 times as potent than the R-(+) isomer (Arrowsmith et al., 1986) in reducing blood pressure via blockade of L-type Ca²⁺ channels. For nicardipine, the R-(+) isomer is ~5 times as potent as the S-(–) isomer (Iwatsuki et al., 1984). All experiments were performed at room temperature (20–23° C).

2.5 Data Analysis

Data were analyzed using clampfit (Axon Instruments, Foster City, CA) and SigmaPlot (SPSS, Chicago, IL) software. IC₅₀ values from dose response curves were determined by fitting the curves to the equation: Relative Current = $1 - \{1/[1 + (IC_{50}/[drug])]\}$. Statistical significance was determined using Student's unpaired t-test or One-way ANOVA. $P < 0.05$ was considered significant.

3. Results

3.1 Closed-Channel Block

A common characteristic of DHP block of L-type channels is that channels in the closed conformation can bind drug, and thus are prevented from opening upon subsequent depolarization (Lee and Tsien, 1983). We determined the IC₅₀ values of amlodipine and

nicardipine for block of closed $\text{Ca}_v1.2$, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive, and $\text{Ca}_v2.1$ channels using a low-frequency stimulation protocol. Cells expressing the indicated channels were held at -60 mV ($\text{Ca}_v1.2$ and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive) or -80 mV ($\text{Ca}_v2.1$) and the membrane potential was stepped to $+10$ mV for 100 ms once every 20 seconds (0.05 Hz). After a steady baseline of current was established, the indicated concentrations of drug were applied to the cell in extracellular solution until a new steady-state level of current was achieved. The fraction of current in the absence of drug remaining in the presence of each drug concentration was plotted against the drug concentration, and IC_{50} values were determined as described in **Materials and Methods**. The dose-dependence of block of the $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive channel by nicardipine and amlodipine is shown in Fig. 2A. Nicardipine blocks $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive in a dose dependent manner ($\text{IC}_{50} = 2.0 \pm 0.1$ μM ; $n = 4$), however, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive is much less sensitive to amlodipine ($\text{IC}_{50} = 26 \pm 3.8$ μM ; $n = 4$). The potency of amlodipine block of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive is not different than that of the neutral DHP isradipine (Fig. 2A; $\text{IC}_{50} = 33 \pm 3.6$ μM ; $n = 4$). In contrast, both amlodipine and nicardipine block $\text{Ca}_v1.2$ channels at nanomolar concentrations (amlodipine $\text{IC}_{50} = 57 \pm 22$ nM; $n = 4$; nicardipine $\text{IC}_{50} = 60 \pm 29$ nM; $n = 4$), and $\text{Ca}_v2.1$ at low micromolar concentrations (amlodipine $\text{IC}_{50} = 8.6 \pm 0.6$ μM ; $n = 4$; nicardipine $\text{IC}_{50} = 7.5 \pm 4.0$ μM ; $n = 4$; Fig. 2B). Thus, while amlodipine and nicardipine are similar in the concentration-dependence of block of both $\text{Ca}_v1.2$ and $\text{Ca}_v2.1$, they differ in their ability to block the $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive under conditions favoring closed-channel block.

Since our data suggest that amlodipine and nicardipine may interact differently with $\text{Ca}_v1.2$, we examined the ability of both drugs to block the mutant $\text{Ca}_v2.1$ channel, $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive (Hockerman et al., 1997b). $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive includes nine amino acid substitutions that confer high sensitivity to the neutral DHP antagonist isradipine, and the DHP agonist $(-)$ -BayK 8644. In these experiments, 100 ms depolarizations to $+10$ mV were applied at 0.05 Hz, from a holding potential of -120 mV. This very negative holding potential was necessary because of the very negative $V_{1/2}$ inactivation of the $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive channel. Representative traces recorded from cells expressing $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive show that nicardipine and amlodipine block this channel in a dose-dependent manner (Fig. 3A and 3B, respectively). Fits of the dose-response data gave IC_{50} values of 41 ± 89 nM and 175 ± 41 nM for amlodipine and nicardipine block of $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive (Fig. 3C). However, this apparent difference in potency of $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive block by amlodipine and nicardipine did not reach statistical significance. As highlighted in Fig. 3D, amlodipine and nicardipine have similar potencies at $\text{Ca}_v1.2$ and $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive channels, but are significantly less potent in blocking $\text{Ca}_v2.1$ channels compared to both $\text{Ca}_v1.2$ and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive. Both amlodipine and nicardipine were significantly more potent in blocking $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive than in blocking $\text{Ca}_v2.1$. While the IC_{50} values of amlodipine and nicardipine were not different for block of $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive, the slope of the dose/response curve was much less steep for amlodipine than for nicardipine (0.48 for amlodipine vs. 0.93 for nicardipine). This difference in slope resulted in a significantly greater percentage of $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive block at 20 nM amlodipine than at 50 nM nicardipine ($52 \pm 3\%$ vs $73 \pm 5\%$ current remaining, respectively; $P < 0.01$). Thus, at concentrations below the IC_{50} , amlodipine blocks $\text{Ca}_v2.1/\text{Dihydropyridine}$ sensitive more potently than nicardipine.

3.2 Frequency-Dependence of Block

Neutral DHP drugs such as isradipine or nitrendipine exert little frequency-dependent block of $\text{Ca}_v1.2$ channels, but block of $\text{Ca}_v1.2$ by the charged drugs diltiazem and verapamil is markedly frequency-dependent (Lee and Tsien, 1983). Therefore, we examined the ability of

both amlodipine and nicardipine to block $\text{Ca}_v1.2$, $\text{Ca}_v2.1$, and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive in a frequency-dependent manner. After drug block at the indicated concentrations reached equilibrium at 0.05 Hz as described above, we applied a 20-pulse, 1-Hz train of 100 ms depolarizations to +10 mV from a holding potential of -80 mV ($\text{Ca}_v2.1$) or -60 mV ($\text{Ca}_v1.2$ and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive) (Fig. 4). Thus, the increase in block detected during the 1-Hz train is due solely to the increase in stimulation frequency. Normalized current during the 1 Hz train in both the absence and presence of drug is shown for comparison (mean $\pm$ SE; $n = 3-5$). As shown in Fig. 4A, 50 nM nicardipine, but not 100 nM amlodipine, exhibited increased block of $\text{Ca}_v1.2$ upon increasing the stimulation frequency. Similarly, the same protocol led to a significant increase in $\text{Ca}_v2.1$ channel block by 10 μM nicardipine but not 10 μM amlodipine at the end of the 1 Hz train of depolarizations (Fig. 4B). Finally, the block of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive by 1 μM nicardipine or 30 μM amlodipine was significantly enhanced upon increasing the frequency of stimulation (Fig. 4C). Thus, for all three channels tested, nicardipine exerted some degree of frequency-dependent block, while amlodipine blocked only $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive in a frequency-dependent manner.

3.3 Block of Inactivated Channels

Since nicardipine and amlodipine blocked some of the channels in a frequency-dependent manner, we examined the ability of both drugs to interact with inactivated $\text{Ca}_v1.2$, $\text{Ca}_v2.1$, and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive channels. As shown in Fig. 5, we applied 1 s depolarizations to +10 mV from a holding potential of -60 mV ($\text{Ca}_v1.2$, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive) or -80 mV ($\text{Ca}_v2.1$) before and after the channels were equilibrated with the indicated concentration of amlodipine or nicardipine. In the absence of drug (control traces, Fig. 5A,B,D), the intrinsic inactivation rate can be measured ($n = 3-6$). For $\text{Ca}_v2.1$, inactivation follows a single exponential time constant of 395 ± 38 ms (Fig. 5, C). Application of 10 μM nicardipine or 10 μM amlodipine significantly accelerated inactivation of $\text{Ca}_v2.1$ during the 1 s depolarization (amlodipine $\tau = 220 \pm 40$ ms, nicardipine $\tau = 137 \pm 21$ ms; Fig. 5A). For $\text{Ca}_v1.2$ in the presence of the β_{1b} subunit, inactivation follows a bi-exponential time course, with a fast and a slow time constant (see (Dilmac et al., 2003)). We found that the application of 100 nM amlodipine did not appreciably change either the fast time constant, the fraction of $\text{Ca}_v1.2$ channels inactivating with the fast time constant, or the slow time constant (Fig. 5B). In contrast, application of 100 nM nicardipine induced a single time constant of inactivation in $\text{Ca}_v1.2$ that was faster than the slow time constant in the absence of drug (Fig. 5E; $\tau = 553 \pm 8$ ms (control) and 318 ± 37 ms (100 nM nicardipine)). Like $\text{Ca}_v1.2$, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive inactivated with a fast ($\tau_f = 90 \pm 11$ ms) and slow ($\tau_s = 756 \pm 114$ ms) time constant, with the slow time constant being by far most prominent (Fig. 5E,F). As in $\text{Ca}_v1.2$, nicardipine (1 μM) induced a single time constant of inactivation in $\text{Ca}_v1.2/\text{DHP}$, that appeared faster than the slow time constant in the absence of drug, but this difference did not reach significance (Fig. 5F). In contrast, 30 μM amlodipine induced a shift to predominantly fast inactivation ($\tau = 56 \pm 9$ ms; Fig. 5E,F) that was indistinguishable from the fast phase of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive inactivation in the absence of drug. Amlodipine also significantly accelerated the slow time constant of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive inactivation ($\tau_s = 263 \pm 113$ ms) (Fig. 5F). We compared the effect of amlodipine on inactivation kinetics in $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive to those of isradipine, a compact neutral dihydropyridine L-type Ca^{2+} channel blocker. We found that 50 μM isradipine caused significantly greater fraction of channels to inactivate with the fast time constant, and induced a significant acceleration of the slow time constant of inactivation, in a manner that was indistinguishable from amlodipine (Fig. 5D, E, F).

Another indication of drug interaction with inactivated channels is a drug-induced leftward shift in the steady-state inactivation curve (Li et al., 1999). Therefore, we also measured the voltage-dependence of inactivation for Ca_v1.2, Ca_v1.2/Dihydropyridine insensitive, and Ca_v2.1 channels in the absence and presence of amlodipine and nicardipine (n = 3–4) (Fig. 6). For Ca_v1.2 channels, 100 nM nicardipine caused a –33 mV shift in $V_{1/2}$ inactivation, while 100 nM amlodipine did not significantly affect $V_{1/2}$ inactivation (Fig. 6A). For Ca_v2.1, 10 μ M amlodipine caused a –13 mV shift in $V_{1/2}$ inactivation while 10 μ M nicardipine shifted $V_{1/2}$ inactivation by only –4 mV (Fig. 6B). For Ca_v1.2/Dihydropyridine insensitive, 1 μ M nicardipine shifted $V_{1/2}$ inactivation by approximately –35 mV, while 30 μ M amlodipine had no significant effect on inactivation (Fig. 6C). Thus, nicardipine interacts strongly with the inactivated $V_{1/2}$ state of both Ca_v1.2 and Ca_v1.2/Dihydropyridine insensitive, but only weakly with the inactivated state of Ca_v2.1. Conversely, amlodipine has no ability to interact with the inactivated state of either Ca_v1.2 or Ca_v1.2/Dihydropyridine insensitive, but can induce a modest leftward shift in $V_{1/2}$ inactivation in Ca_v2.1. The $V_{1/2}$ inactivation and k values derived from the data shown in Fig. 6 are given in Table 1.

4. Discussion

4.1 Closed-channel Block of Ca_v1.2 by Amlodipine and Nicardipine

In this study, we tested the hypothesis that amlodipine and nicardipine have distinct interactions with the L-type Ca²⁺ channel Ca_v1.2 as a result of the distinct orientation of their alkylamino side chains. Both drugs block Ca_v1.2 with nearly identical potencies at low frequency stimulation which favors block of closed channels. Moreover, the high potency block of closed Ca_v1.2 channels by nicardipine and amlodipine was clearly dependent upon Thr 1039 and/or Gln 1043 as the mutant channel Ca_v1.2/Dihydropyridine insensitive, in which these residues are changed to Tyr and met, respectively, markedly reduces the potency of both drugs. However, the decrease in potency of amlodipine in block of Ca_v1.2/Dihydropyridine insensitive compared to Ca_v1.2 is significantly greater than the decrease in potency in block of the same channels by nicardipine. We compared the potency of nicardipine and amlodipine block of Ca_v1.2/Dihydropyridine insensitive channels to that of isradipine, a neutral DHP drug with no alkylamino side chain and high potency at Ca_v1.2 channels (Hockerman et al., 1997b). The potency of closed-channel block of Ca_v1.2/Dihydropyridine insensitive channels by isradipine and amlodipine was indistinguishable, while nicardipine was clearly more potent in blocking these channels than either isradipine or amlodipine.

Our experiments with the Ca_v2.1/Dihydropyridine sensitive channel further confirms that both amlodipine and nicardipine interact with the well-described neutral DHP site since both block Ca_v2.1/Dihydropyridine sensitive at much lower concentrations than Ca_v2.1, and at concentrations similar to those that block Ca_v1.2. While the IC₅₀ values for amlodipine and nicardipine block of Ca_v2.1/Dihydropyridine sensitive were not significantly different, amlodipine did clearly block a greater percentage of Ca_v2.1/Dihydropyridine sensitive current at sub-IC₅₀ concentrations than nicardipine. This observation, combined with the finding that the IC₅₀ for nicardipine block of Ca_v1.2/Dihydropyridine insensitive channels is significantly lower than that of amlodipine, and the clear interaction of nicardipine with the inactivated state of the Ca_v1.2 channel, suggests that nicardipine may interact with amino acid residues outside of the canonical DHP binding site, while amlodipine does not.

Our observation, that both nicardipine and amlodipine block Ca_v1.2/Dihydropyridine insensitive with differing potencies, is consistent with an earlier study that compared the effect of several mutations within the DHP binding site on a pair of DHP derivatives that differed only by the presence (DHPch) or absence (DHPn) of a quaternary amino group (Lacinova et al., 1999). In their study, Lacinova et al. found that one of the mutations

included in $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive (the Thr1039 to Tyr substitution) markedly reduced block by DHPn, but retained sensitivity to block by the DHPch. They concluded that the presence of the charged substituent allowed interaction with distinct binding determinants within the Thr1039Tyr $\text{Ca}_v1.2$ mutant. Interestingly, the position on the DHP ring of the charged alkylamino side chain in DHPch corresponds to that of the aminoalkyl side chain of nifedipine. Taken together, our results suggest that the position of the alkylamino substituent on the DHP ring impacts the manner in which amlodipine and nifedipine interact with these binding determinants outside of transmembrane domain IIIS5, and that the alkylamino side chain of nifedipine, but not amlodipine, is likely to project out of the canonical DHP binding site, and contribute to potency of closed-channel block of $\text{Ca}_v1.2$.

The IC_{50} values that we report for closed-channel block of $\text{Ca}_v1.2$ are in reasonable agreement with several studies using nifedipine (Kuga et al., 1990) and amlodipine (Burgess et al., 1987) (Kass and Arena, 1989) in mammalian cells. In contrast, a study that utilized the *Xenopus* oocyte expression system reported low micromolar IC_{50} values for block of $\text{Ca}_v1.2$ by both amlodipine and nifedipine (Furukawa et al., 1999). The large difference in the sensitivity of $\text{Ca}_v1.2$ to amlodipine and nifedipine between the present study and that of Furukawa et al., could be the result of decreased sensitivity of channels expressed in the oocyte expression system compared to expression in mammalian cells.

4.2 Frequency dependent block of $\text{Ca}_v1.2$ by amlodipine and nifedipine

We further explored the differences between nifedipine and amlodipine by testing the frequency-dependence of their block of $\text{Ca}_v1.2$, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive, and $\text{Ca}_v2.1$. Previous studies with amlodipine suggested that block and unblock by the charged species is slower than for the neutral species, but that increasing stimulation frequency does not enhance amlodipine block (Kass et al., 1989; 1991). Indeed, we found that amlodipine block of $\text{Ca}_v1.2$ was not enhanced by increasing the frequency of stimulation from 0.05 Hz to 1 Hz. However, amlodipine does exhibit marked frequency-dependent block and accelerate apparent inactivation in $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive, but does not significantly shift steady-state inactivation. These characteristics suggest that amlodipine is an open channel blocker, and does not appreciably stabilize the inactivated state of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive. The observation that isradipine blocks $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive in a manner indistinguishable from amlodipine supports our proposal that amlodipine blocks this mutant channel in the open state, since isradipine was previously reported to block a similar dihydropyridine-resistant $\text{Ca}_v1.2$ channel at high concentrations via the open channel (Lacinova and Hofmann, 1998). In contrast, the marked negative shift in $V_{1/2}$ inactivation for $\text{Ca}_v1.2$ and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive and lack of a strong effect on the kinetics of inactivation in the presence of nifedipine suggests that the frequency-dependent block by nifedipine in these channels may be the result a slowing of recovery from inactivation. However, previous studies concluded that amlodipine blocks L-type channels in a weakly frequency-dependent manner (Kass and Arena, 1989) (Hughes and Wijetunge, 1993), and that it induces a negative shift in $V_{1/2}$ inactivation of L-type vascular smooth muscle cells (Hughes and Wijetunge, 1993). Nifedipine was previously shown to shift the voltage-dependence of inactivation of L-type channels in tracheal smooth muscle cells by -12 mV at an IC_{50} concentration (Yamakage et al., 1997), in reasonable agreement with the present study. Thus, it is possible that species or splice variations in the $\text{Ca}_v1.2 \alpha_1$ subunit, the sub-type of auxiliary subunits, or the cellular environment can affect the ability of nifedipine and amlodipine to interact with the inactivated state of L-type channels.

4.3 Block of Ca_v2.1 by amlodipine and nicardipine

It is clear that both amlodipine and nicardipine effectively block Ca_v2.1 channels at low micromolar concentrations (present study), while neutral dihydropyridines such as isradipine (Hockerman et al., 1997b), nifedipine, nitrendipine, and nimodipine (Furukawa et al., 1999) do not appreciably block Ca_v2.1 channels at concentrations up to 10 μM. It is possible that block of non-L-type channels by amlodipine and nicardipine may be relevant to the pharmacological profile of these drugs. Further characterization of the block of Ca_v2.1 by nicardipine suggests that it interacts with the inactivated state of Ca_v2.1 channels since it blocks in a frequency-dependent manner, accelerates current decay during a 1 s depolarization, and shifts the voltage-dependence of inactivation to more negative potentials. In the present study, nicardipine blocked Ca_v2.1 current at significantly lower concentrations than those reported by Furukawa et al., who expressed a similar Ca_v2.1 clone in *Xenopus* oocytes (Furukawa et al., 1999). However, the IC₅₀ value for block of Ca_v2.1 by amlodipine reported in this study is virtually identical to that reported by Furukawa et al.. A recent study (Miyashita et al., 2010) reported that amlodipine blocks Ca_v2.2 at low micromolar concentrations, and that this block is disrupted by mutation of a Met residue in IIS5 to Lys. Thus, even in the Ca_v2.2 channel which is devoid of the canonical DHP binding site found in Ca_v1.2 channels, amlodipine appears to interact strongly with amino acid residues in domain IIS5, suggesting that amlodipine interacts predominantly with amino acid residues within a binding pocket formed by transmembrane domains IIS5, IIS6, and IVS6.

4.4 Orientation of alkylamino side chains of amlodipine and nicardipine

Our results are consistent with two recent studies that examined the interaction of nicardipine and amlodipine with Ca_v1.2. A study examining mutations in the IIS6 region of Ca_v1.2 found that mutation of Ala752, located on the intracellular side of IIS6, to Thr selectively reduced the potency of nicardipine block compared to the neutral dihydropyridines, nifedipine and nimodipine (Hui et al., 2009). This suggests that nicardipine may well interact with amino acid residues in transmembrane domain IIS6 to account for the increased potency of nicardipine over amlodipine in block of Ca_v1.2/Dihydropyridine insensitive. Such an interaction would support our assertion that the alkylamino side chain of nicardipine projects into the pore and contributes to both the potency of closed-channel block and to the state-dependence of block of Ca_v1.2. In contrast, a study using *in silico* models of Ca_v1.2 structure (Cosconati et al., 2007) proposed that the alkylamino side chain of amlodipine folds back toward the DHP ring and interacts via H-bonding with the same amino acid residue in transmembrane domain IIS5 (Gln1043) as the proton of the DHP nitrogen atom. Given that Gln1043 is one of the two amino acids mutated to generate the Dihydropyridine insensitive mutation, this model is supported by our data indicating that amlodipine block of closed Ca_v1.2/Dihydropyridine insensitive channels is not different from that of isradipine. It will be of interest to further define the interactions of both amlodipine and nicardipine with Ca_v1.2 as these data are likely to yield insight into the orientation of the DHP ring within its binding site.

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Fig. 1. Structural Features of the α_1 subunit of Ca_v Channels, Amlodipine and Nicardipine

A. The structure of amlodipine and nicardipine. Note that the ionizable alkylamino groups are on opposite sides of the DHP ring relative to the pseudoaxial 4-aryl group. **B.** Topology of Ca_v Channels. Shaded cylinders represent transmembrane segments (1–6) organized into four homologous domains (I–IV). The C- and N-terminal domains are intracellular. The position of transmembrane domain IIIS5 is indicated. The alignment of transmembrane domain IIIS5 amino acids residues for $Ca_v1.2$, $Ca_v1.2$ /Dihydropyridine insensitive, and $Ca_v2.1$ is shown. Dashes represent identity between all three channels. Bold letters indicate the amino acids inserted into $Ca_v1.2$ /Dihydropyridine insensitive. The L-type channel $Ca_v1.2$ /Dihydropyridine insensitive is a double mutant that substitutes the $Ca_v2.1$ amino acids in positions 1039 and 1043 (T 1039 to Y + Q 1043 to M). $Ca_v1.2$ /Dihydropyridine insensitive is highly insensitive to neutral dihydropyridines such as isradipine, nifedipine, and nisoldipine but retains normal sensitivity to the benzothiazepine diltiazem (Hockerman et al., 2000; Liu et al., 2003; Walsh et al., 2007). Fig. 1B shows the amino acid alignments of all three transmembrane domains containing the amino acid residues critical for DHP block, IIIS5, IIIS6, and IVS6, from $Ca_v2.1$ and $Ca_v1.2$. The $Ca_v1.2$ -specific residues indicated were inserted into $Ca_v2.1$ to construct the mutant channel $Ca_v2.1$ /Dihydropyridine sensitive. **B.** The amino acid sequences for transmembrane segment IIIS5, IIIS6, and IVS6 in wild type $Ca_v2.1$, $Ca_v2.1$ /Dihydropyridine sensitive, and WT $Ca_v1.2$ are aligned. The nine amino acids in bold font were inserted into $Ca_v2.1$ to create $Ca_v2.1$ /Dihydropyridine sensitive.

Fig. 2. Block of $\text{Ca}_v1.2$, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive, and $\text{Ca}_v2.1$ by amlodipine and nicardipine at 0.05 Hz

A, Dose-response relationship for block of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive by nicardipine (closed circles), amlodipine (closed triangles), and isradipine (open circles). The averaged, normalized current amplitudes at the indicated drug concentrations are shown $\pm$ S.E. ($n = 4$). The IC_{50} values were determined by fitting the data to the equation, $\text{relative current} = 1 - \{1/[1 + (\text{IC}_{50}/[\text{drug}])]\}$ (smooth line). Cells were held at -60 mV, and depolarized to $+10$ mV for 100 ms every 20 s (0.05 Hz). The IC_{50} value for isradipine block of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive was 33 ± 3.6 μM . **B**, the IC_{50} values of nicardipine and amlodipine block of the indicated channels are shown $\pm$ S.E. ($n = 4$). Voltage-dependent barium current was elicited using the same pulse protocol as in **A**, except that a holding potential of -80 mV was used in experiments with $\text{Ca}_v2.1$ channels. The values were: for nicardipine, $\text{Ca}_v1.2 = 60 \pm 29$ nM, $\text{Ca}_v2.1 = 7.5 \pm 3.9$ μM , $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive = $2.0 \pm .07$ μM ; for amlodipine, $\text{Ca}_v1.2 = 57 \pm 22$ nM, $\text{Ca}_v2.1 = 8.6 \pm 0.58$, $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive = 26.0 ± 3.8 μM . ***, $P < 0.001$, (One-way ANOVA, Holm-Sidak post hoc test) compared to nicardipine block of $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive.

Fig. 3. Block of $\text{Ca}_v2.1$ /Dihydropyridine sensitive by Amlodipine and Nicardipine at 0.05 Hz
Barium currents were measured as described in Fig. 2 except that the holding potential was -120 mV. **A–B**, Representative $\text{Ca}_v2.1$ /Dihydropyridine sensitive current traces recorded in the absence (control) or presence of the indicated concentrations of nicardipine (**A**) or amlodipine (**B**). **C**, Dose-response relationships for amlodipine (closed squares) and nicardipine (open squares) block of $\text{Ca}_v2.1$ /Dihydropyridine sensitive. Smooth lines represent fits of the data as in Fig. 2. The percentage of $\text{Ca}_v2.1$ /Dihydropyridine sensitive current blocked by 20 nM amlodipine is significantly greater than that blocked by 50 nM nicardipine (*, $P < 0.05$; Student's unpaired t-test). **D**, Comparison of the IC_{50} values for amlodipine and nicardipine block of $\text{Ca}_v1.2$, $\text{Ca}_v2.1$, and $\text{Ca}_v2.1$ /Dihydropyridine sensitive. The holding potentials used for experiments with $\text{Ca}_v1.2$ and $\text{Ca}_v2.1$ were -60 mV and -80 mV, respectively. IC_{50} values for nicardipine and amlodipine block of $\text{Ca}_v2.1$ /Dihydropyridine sensitive were 174 ± 40 nM and 41 ± 89 nM, respectively. Data shown are mean values $\pm$ SE ($n = 5$). IC_{50} values for $\text{Ca}_v1.2$ and $\text{Ca}_v2.1$ are from Fig. 2. The IC_{50} values of both amlodipine and nicardipine for block of $\text{Ca}_v2.1$ are significantly different from the IC_{50} values for block of both $\text{Ca}_v1.2$ (###, $P < 0.001$) and $\text{Ca}_v2.1$ /Dihydropyridine sensitive (***, $P < 0.001$). (One-way ANOVA with Holm-Sidak post hoc test)

Fig. 4. Block of $\text{Ca}_v1.2$, $\text{Ca}_v2.1$, and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive by Amlodipine and Nicardipine at 1 Hz

A–C, Whole-cell Ba^{2+} currents were recorded in the absence and presence of 100 nM amlodipine or 500 nM nicardipine ($\text{Ca}_v1.2$), 10 μM amlodipine or 10 μM nicardipine ($\text{Ca}_v2.1$), and 1 μM nicardipine and 30 μM amlodipine ($\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive) using 100 ms depolarizations to +10 mV from a holding potential of –60 mV ($\text{Ca}_v1.2$ and $\text{Ca}_v1.2/\text{Dihydropyridine}$ insensitive) or –80 mV ($\text{Ca}_v2.1$), at a frequency of 1 Hz. Current measured in the presence of drug followed equilibration of block by the indicated concentration at 0.05 Hz. Relative peak current (mean $\pm$ S.E.) in each successive depolarizing pulse is plotted against pulse number ($n = 3\text{--}5$) in the absence (closed symbols) or presence (open symbols) of drug. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; Student's unpaired t-test comparing the 20th pulse in the presence of drug to the 20th pulse in the absence of drug.

Fig. 5. Time Course of Amlodipine and Nicardipine Block of Depolarized Channels

A–B, Representative traces of Ba²⁺ current through WT Ca_v2.1 and Ca_v1.2 channels measured during a 1 s depolarization to +10 mV from a holding potential of –80 mV (Ca_v2.1) in the absence (control) or presence of the indicated concentration of drug. Current measured in the absence and presence of drug is normalized to peak current to facilitate comparison of the rate of current decay. **C**, Time constants (τ) for inactivation in the absence (Control: gray bars) or presence of the indicated concentrations of amlodipine (white bars) or nicardipine (cross-hatched bars) for the indicated channel. For Ca_v2.1, inactivation follows a single exponential time constant. For Ca_v1.2, inactivation follows two exponential time constants. Data shown for Ca_v1.2 represents the slow time constant, which is by far the most prominent. Ca_v2.1: τ = 395 $\pm$ 38 ms in the absence of drug, τ = 220 $\pm$ 40 ms in the presence of 10 μ M amlodipine, τ = 137 $\pm$ 21 ms in the presence of 10 μ M nicardipine. Ca_v1.2: τ = 595 $\pm$ 50 ms in the absence of drug, τ = 541 $\pm$ 7 ms in the presence of 100 nM amlodipine, τ = 318 $\pm$ 37 ms in the presence of 100 nM nicardipine. **D**, Representative traces of Ba²⁺ currents through Ca_v1.2/Dihydropyridine insensitive at 10 mV from a resting potential of –60 mV in the presence or absence of the indicated drugs. **E**, Fraction of current inactivating with the fast time constant in Ca_v1.2/Dihydropyridine insensitive channels in the absence and presence of 30 μ M amlodipine or 50 μ M isradipine. Control: 0.21 $\pm$ 0.06 (n = 13); amlodipine: 0.74 $\pm$ 0.23 (n = 6); isradipine: 0.67 $\pm$ 0.14 (n = 4). **F**, Time constants of inactivation for Ca_v1.2/Dihydropyridine insensitive in the absence or presence of 30 μ M amlodipine, 50 μ M isradipine, or 1 μ M nicardipine. Control: τ _{fast} = 89 $\pm$ 11 ms (n = 13), τ _{slow} = 756 $\pm$ 113 ms (n = 12); amlodipine: τ _{fast} = 56 $\pm$ 8 ms (n = 6), τ _{slow} = 263 $\pm$ 113 ms (n = 4); isradipine: τ _{fast} = 70 $\pm$ 19 ms (n = 4), τ _{slow} = 263 $\pm$ 60 ms (n = 4); nicardipine: τ = 540 $\pm$ 99 ms (n = 3). Results shown are mean $\pm$ S.E. of individual fits. Asterisks indicate significant differences from control (* p < 0.05, ** p < 0.01, *** p < 0.001; One-way ANOVA with either Dunnett's or Tukey's post hoc test).

Fig. 6.

Modulation of Voltage-Dependent Inactivation in $Ca_v1.2$, $Ca_v2.1$, and $Ca_v1.2$ /Dihydropyridine insensitive by Amlodipine or Nicardipine.

Peak Ba^{2+} current elicited by depolarization to +10 mV immediately after 10 s conditioning pulses to the indicated potentials from a holding potential of -80 mV ($Ca_v1.2$, $Ca_v1.2$ /Dihydropyridine insensitive) or -100 mV ($Ca_v2.1$) are plotted against the conditioning pulse voltage (mean $\pm$ S.E., $n = 3-4$) in the absence (closed circles) or presence of the indicated concentration of amlodipine (closed triangles) or nicardipine (open circles). The data are fit (smooth lines) to the equation, $Relative\ Current = 1 / \{1 + \exp[(V - V_{1/2})/k]\}$, where V is the conditioning potential, $V_{1/2}$ is the voltage at which half of the channels are inactivated, and k is a slope factor (potential required for an e -fold change). **A**, Steady-state inactivation of $Ca_v1.2$ in the absence of drug (control), in presence of 100 nM amlodipine, or the presence of 100 nM nicardipine. **B**, Steady-state inactivation of $Ca_v2.1$ in the absence of drug in the presence of 10 μ M amlodipine, or in the presence of 10 μ M nicardipine. **C**, Steady-state inactivation of $Ca_v1.2$ /Dihydropyridine insensitive in the absence of drug, in the presence of 1 μ M nicardipine or in the presence of 30 μ M amlodipine.

Table 1

Modulation of voltage-dependence of inactivation

	Control	Amlodipine	Nicardipine
Ca _v 1.2		100 nM	100 nM
<i>V</i> _{1/2 inact.}	-13.2 ± 0.7 mV	-15.7 ± 0.4 mV	-46.5 ± 0.6 mV ^a
<i>K</i>	-8.1 ± 0.6	-7.4 ± 0.4	-4.9 ± 0.5 mV ^b
Ca _v 2.1		10 μM	10 μM
<i>V</i> _{1/2 inact.}	-55.0 ± 0.5 mV	-68.0 ± 1.1 mV ^a	-59.4 ± 1.0 mV ^b
<i>K</i>	-8.4 ± 0.4	-10.5 ± 1.0	-8.6 ± 0.9
Ca _v 1.2/Dihydropyridine insensitive		30 μM	1 μM
<i>V</i> _{1/2 inact.}	-10.0 ± 0.6 mV	-8.8 ± 0.6 mV	-47.5 ± 1.0 mV ^a
<i>K</i>	-6.3 ± 0.6	-4.9 ± 0.5	-6.1 ± 0.9

^a, P<0.001;^b, P< 0.05; One-way ANOVA, Tukey's post hoc test (n = 3–4).