Influence of permeating ions on Kv1.5 channel block by nifedipine

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NIFEDIPINE HAS BEEN WIDELY USED for almost two decades in the control of cardiac chest pain and hypertension, but has been associated, in a dose-dependent manner, with unfavorable side effects like negative inotropy and hypotension, proarrhythmia, and (in some studies) increased mortality. This may occur especially with administration of short-acting forms of nifedipine in patients who are already hemodynamically compromised (13, 21). Part of the mechanism for proarrhythmia caused by nifedipine may be the block of myocardial K⁺ channels, which has been described both in mammalian cardiac myocytes with equilibrium dissociation constants (Kₐ) of 0.5–1 µM and in cloned channels (12, 32). The plateau phase of the cardiac action potential is normally terminated by repolarizing outward potassium fluxes. Therefore, nifedipine block can prolong the action potential, cause a dispersion of refractoriness as these channels differ in their regional distribution across the myocardial wall (18), and lead to instability of the resting potential of the muscle.

Despite the potential importance of these K⁺ channel actions of nifedipine, the mechanisms by which it exerts its actions on K⁺ channels are not fully defined. Many compounds block K⁺ channel currents by binding at or near the internal or external mouth of the ion-conducting pore, where they may act as direct open channel blockers by physically occluding the ion-conducting pore to prevent ion passage. Examples of this kind of blocker are quinidine and the quaternary ammonium compounds like tetraethylammonium (TEA), as well as the NH₂-terminal inactivation particle in Shaker channels (8). It is known that nifedipine acts as an open channel blocker of single Shaker mutant channels (1) and of the mammalian homolog Kv1.5 channels with a Kᵣ of 6.3 µM (32). Whether nifedipine acts at the internal or external mouth of the K⁺ channel pore, though, is presently uncertain. Other details of the action of permeant ions on nifedipine block and the ability of the drug to bind to channel-inactivated states are also poorly understood. Kv channels show a rapid N-type inactivation or a slower C-type inactivation, affected by factors such as elevation of the extracellular K⁺ concentration ([K⁺]₀) (2, 20), external application of TEA (6, 11), and mutations of particular amino acids in the channel pore (7, 20, 24). These effects have been incorporated into a physical model where C-type inactivation is caused by the constriction of the outer mouth of the channel pore in a cooperative action of all four subunits (30), which restricts K⁺ flux (23, 24). This rearrangement of the outer mouth of the pore greatly reduces the permeability of K⁺ relative to the permeability of Na⁺, altering the ion selectivity of the channel (27). Recently, it has been further proposed that, during C-type inactivation, the channels dwell in at least three conformational states: an initial open state that is highly selective for K⁺, a state that is less permeable to K⁺ and more permeable to Na⁺, and then a state that is nonconducting (17, 19).
Kv1.5 is a prominent cardiac K⁺ channel α-subunit because it is expressed in the human heart, particularly in the human atrium, and possibly in other regions of the human cardiovascular system (9, 28). Nifedipine blocks Kv1.5 as an open channel blocker with a Ki of 6.3 μM (32). Here, we present evidence that the nature of the permeant ion does affect the dose dependence and kinetics of nifedipine block of Kv1.5 channels. This suggests that nifedipine block involves coupling between permeation and the blocking site and localizes the binding of nifedipine within the permeation pathway of K⁺ channels. Outer pore mutations of R487 reduce the affinity for Kv1.5 channel block in manner consistent with other channels that have a valine residue at equivalent sites. We also show that nifedipine block, although affected by mutations similar to those known to affect outer pore C-type inactivation, can be shown to be kinetically separate from this slow inactivation process. This is based on nifedipine block of Kv1.5 carried out in different [K⁺]o, and also on the effects of nifedipine on the recovery rates from C-type inactivation.

MATERIALS AND METHODS

Cell culture. Human Kv1.5 channels stably expressed in HEK-293 cell lines were used in all experiments. Kv1.5 in the plasmid expression vector pCDNA3 was mutagenized using the Quickchange Kit (Stratagene, La Jolla, CA) to convert arginine residue 487 to valine (R487V) or tyrosine (R487Y). HEK-293 cells were stably transfected with wild-type Kv1.5 or Kv1.5-R487(V/Y) cDNAs using LipofectACE reagent (Canadian Life Technologies, Bramalea, ON, Canada) in a 1-to-10 (wt/vol) ratio. Patch pipettes contained (in mM) 135 KCl, 5 EGTA, and 10 HEPES and were adjusted to pH 7.4. For recordings in the presence of different external Cs⁺, Rb⁺, or K⁺ concentrations, the NMG⁺ base external solution was used, and KCl was substituted by the appropriate ions. The elevated external K⁺ solutions contained (in mM) 5, 135, or 300 KCl, 10 HEPES, and 1 CsCl; pH 7.4. Nifedipine was dissolved in ethanol at a stock concentration of 20 mM and inactivated external K⁺ and was adjusted to pH 7.4 with HCl. For nifedipine block of Kv1.5 carried out in different [K⁺]o, the pH was adjusted with CsOH, RbOH, or HCl, respectively. The base bath solution contained (in mM) 135 NMG⁺, 5 KCl, 10 HEPES, 1 MgCl₂, and 1 CaCl₂ and was adjusted to pH 7.2 with NaOH. The contaminating Cl⁻ concentration was 99–100.5% (by HCl titration, Sigma M2004). All water used in these experiments was passed through organic filters and two-stage distillation before a Milli-Q (Millipore) deionizing system returned the water at a specific resistance of ~20 MΩ. The contaminating K⁺ in the water used for solutions was below detection limits (<0.25 μM) for coupled plasma optical emission spectroscopy (CANTEST Analytical Services, Vancouver, Canada), and the 140 mM NMG⁺ solution also had undetectable levels of K⁺. The 135 mM Na⁺ solution gave a reading of 9.5 μM K⁺ due to interference by the high Na⁺ concentration.

Electrophysiological procedures. Coverslips containing cells were removed from the incubator before experiments and placed in a superfusion chamber (volume 250 μl) containing the control bath solution at 22–23°C. The bath solution was exchanged by switching the perfusates at the inlet of the chamber, with complete bath solution changes taking 5–10 s. Whole cell current recording and data analysis were done using an Axopatch 200A amplifier and pCLAMP version 6.0 software (Axon Instruments, Foster City, CA). Patch electrodes were fabricated using thin-walled borosilicate glass (World Precision Instruments; Sarasota, FL). Capacity compensation and 80–95% series resistance compensation were routinely used. The averaged cell membrane capacitance was 15.1 ± 0.5 pF, n = 128, and measured series resistance was between 0.5–5.5 MΩ for all recordings (averaged series resistance was 2.3 ± 0.1 MΩ, n = 128). When this changed during the course of an experiment, data were discarded. Data were filtered at 5–10 kHz and sampled at 10–20 kHz. Gating currents were recorded as described previously (29). The data for analysis and presentation were off-line leak subtracted if required, and data were discarded if the leakage conductance was >1 nS. Throughout the text, data are shown as means ± SE.

Data analysis. The concentration-response curves for permeating K⁺, Rb⁺, and Cs⁺ ions were computer fitted to the Hill equation

\[ f = \frac{1}{1 + (IC_{50}[D])^{nH}} \]  

where \( f \) is the fractional current (Idrug/Icontrol) at drug concentration \( D \), IC_{50} is the concentration producing half-maximal inhibition, and \( nH \) is the Hill coefficient. The time constant \( (\tau_d) \) of the rapid component of current decay in the presence of nifedipine was used as an approximation of the drug channel interaction kinetics, as described previously (4, 32) according to the equation

\[ 1/\tau_d = k_{+1}[D] + k_{-1} \tag{2a} \]

and

\[ K_d = k_{-1}/k_{+1} \tag{2b} \]

in which \( \tau_d \) is the current decay time constant caused by the drug, \( k_{+1} \) and \( k_{-1} \) are the apparent rate constants of binding and unbinding for the drug, respectively, and \( K_d \) represents the affinity of the drug for its binding site.

The data for recovery from inactivation with a short initial depolarization were fit using a single-exponential equation

\[ y = A \times \exp(-t/\tau) + C \tag{3a} \]

where \( A \) is the amplitude, \( t \) is the distance from the base of the curve, \( \tau \) is the time constant for the rising phase of the curve, and \( C \) is the offset constant. Recovery from C-type inactivation (with a long initial pulse) and nifedipine block were fit using a double-exponential equation

\[ y = A_2 \times \exp(-t/\tau_2) + A_1 \times \exp(-t/\tau_1) + I \tag{3b} \]

in which \( \tau_2 \) and \( \tau_1 \) are the fast and slow time constants of current recovery or block, \( A_2 \) and \( A_1 \) are the corresponding amplitudes, and \( I \) indicates the noninactivating or nifedipine-insensitive current.

Experimental values are given as means ± SE. ANOVA and multiple pairwise comparison were used to test for significant differences between groups (Table 1). An unpaired t-test was used to compare off-gating current reduction with current recovery or block, \( A_2 \) and \( A_1 \), the corresponding amplitudes, and \( I \) indicates the noninactivating or nifedipine-insensitive current.

RESULTS

Nifedipine blocks K⁺, Rb⁺, and Cs⁺ currents through Kv1.5 channels. The currents in Fig. 1 illustrate the actions of nifedipine on K⁺, Rb⁺, and Cs⁺ currents
through Kv1.5 channels. Currents were recorded using a physiological ion gradient across the cell membrane [135 mM intracellular K\(^+\) concentration ([K\(^+\)]\(_i\))/5 mM [K\(^+\)]\(_o\)] or a solution with external and internal K\(^+\) replaced by equimolar Rb\(^+\) or Cs\(^+\). The control traces in Fig. 1A were elicited from a holding potential of \(-80\) mV to voltages between \(-30\) and \(+40\) mV in increments of \(10\) mV. As indicated, K\(^+\) (left), Rb\(^+\) (middle), and Cs\(^+\) (right) currents were recorded in the control (A) and presence of 10 (B) and 50 \(\mu\)M nifedipine (C), respectively. The scale bars in A also apply to B and C, and data for each ion in A–C are from the same cells. D: steady-state current-voltage relations for K\(^+\), Rb\(^+\), and Cs\(^+\) current block by nifedipine from current data above. In the absence (open symbols) and presence of 10 and 50 \(\mu\)M nifedipine (filled symbols), the steady-state K\(^+\) (left), Rb\(^+\) (middle), and Cs\(^+\) (right) currents measured at the end of current traces were plotted against test potential.

Fig. 1. Block of Kv1.5 K\(^+\), Rb\(^+\), and Cs\(^+\) currents in HEK cells by nifedipine. Whole cell currents were elicited from a holding potential of \(-80\) mV to voltages between \(-30\) and \(+40\) mV in increments of \(10\) mV. As indicated, K\(^+\) (left), Rb\(^+\) (middle), and Cs\(^+\) (right) currents were recorded in the control (A) and presence of 10 (B) and 50 \(\mu\)M nifedipine (C), respectively. The scale bars in A also apply to B and C, and data for each ion in A–C are from the same cells. D: steady-state current-voltage relations for K\(^+\), Rb\(^+\), and Cs\(^+\) current block by nifedipine from current data above. In the absence (open symbols) and presence of 10 and 50 \(\mu\)M nifedipine (filled symbols), the steady-state K\(^+\) (left), Rb\(^+\) (middle), and Cs\(^+\) (right) currents measured at the end of current traces were plotted against test potential.

block reduces current amplitude without significantly altering kinetics. External application of 10 \(\mu\)M nifedipine also blocked Rb\(^+\) and Cs\(^+\) currents (Fig. 1B), but the potency of block was significantly less. Application of 50 \(\mu\)M nifedipine (Fig. 1C) produced a more marked block of current carried by all three cations and a significant acceleration of current decay that reflected open channel block caused by the drug. Steady-state current-voltage relations from these data are shown in Fig. 1D for the three ions and show the increased potency of nifedipine action when K\(^+\) was carrying current through Kv1.5 rather than when Rb\(^+\) or Cs\(^+\) were carrying current. At \(+40\) mV, external application of 10 \(\mu\)M nifedipine blocked more than 60% of K\(^+\) current. However, the same concentration of nifedipine only blocked 35% of Rb\(^+\) current and 20% of Cs\(^+\) current. External application of 50 \(\mu\)M nifedipine blocked 86% of K\(^+\) current, 70% of Rb\(^+\) current, and 60% of Cs\(^+\) current. As we (32) have previously noted, nifedipine block was minimal until potentials around \(-10\) mV when channel open probability was significant. At potentials where the channel open probability was high, block was only mildly dependent on pulse.
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The potency of nifedipine block was quantified in the dose-response curves shown in Fig. 2. With the use of a Hill equation to fit curves obtained from measurements of steady-state K⁺, Rb⁺, and Cs⁺ Kv1.5 currents at different concentrations of nifedipine, the resulting IC₅₀ values were 7.3, 16.0, and 26.9 μM with Hill coefficients of 0.9, 1.1, and 1.2 for K⁺, Rb⁺, and Cs⁺, respectively (Fig. 2A). Hill coefficients close to 1.0 suggested a single binding site for nifedipine block of Kv1.5 no matter which ion species was permeating the channel. These results indicated that the potency of nifedipine block of Kv1.5 was not only dependent on nifedipine concentration but also strongly dependent on the permeating-ion species carrying Kv1.5 currents.

One of the noticeable features of the inhibition of Kv1.5 by nifedipine was the concentration-dependent increase in the apparent rate of current inactivation (Fig. 1). In the control, the currents reached their peak at ~10 ms and then declined slowly. During 400-ms voltage pulses at +40 mV, the currents could be fitted to a single exponential function (Eq. 3a) with a decay time constant of 214 ± 17 ms (n = 20), 297 ± 20 ms (n = 12), and 298 ± 29 ms (n = 9) for K⁺, Rb⁺, and Cs⁺, respectively. In the presence of nifedipine, the peak current was reduced and reached at earlier times. The subsequent decay introduced an additional exponential component superimposed on the slow inactivation, so a biexponential function was used to fit the current decay in the presence of nifedipine (Eq. 3b). Between 5 and 50 μM nifedipine, the time constants of the slow component of the current decay (τ₁) are not significantly different from the control values (Table 1), suggesting that the process of slow inactivation was not modified by nifedipine. The time constants of the initial fast-decaying component (τ₂) decrease monotonically as a function of the nifedipine concentration (Table 1). This extra fast component is at least 10 times faster than the slow inactivation. Therefore, the time constant (τ₂) for this fast-decaying component was considered to be a reasonable approximation of the drug-channel interaction kinetics and was used to calculate the Kₐ and on- and off-rates for nifedipine binding according to a single-site model (4). As shown in Fig. 2B, we calculated 1/τ₂ for Kv1.5 channels with the three permeant ions K⁺, Rb⁺, and Cs⁺ and plotted it as a function of nifedipine concentration. The relationship between 1/τ₂ and nifedipine concentration is well fit by Eq. 2a. From the fit lines, we extracted the apparent Kₐ values of 7.8, 16.5, and 27.2 μM for K⁺, Rb⁺, and Cs⁺ block, respectively. These are close to the IC₅₀ values obtained from the concentration-response relations in Fig. 2A and, therefore, support the applicability of a single-binding site model for nifedipine action on Kv1.5 with the three different permeant ions.

Membrane sidedness of cation modulation of nifedipine action. One of the primary aims of the present study was to determine the site of action of nifedipine on the Kv1.5 channels. Because the potency of block varied with different cation species permeating the channel, the following experiments were designed to

Fig. 2. Concentration-dependence of nifedipine block of Kv1.5 K⁺, Rb⁺, and Cs⁺ currents. A: concentration-response curves for nifedipine block of steady-state K⁺, Rb⁺, and Cs⁺ currents [current with nifedipine (Iₙif)/current in control (Icontrol)]. Residual current in nifedipine was measured at the end of 400-ms depolarizations and normalized to current level before nifedipine. Solid lines were fit to the data using a Hill equation (see MATERIALS AND METHODS). The IC₅₀ concentrations for K⁺, Rb⁺, and Cs⁺ currents blocked by nifedipine were 7.3 ± 0.3, 16.0 ± 0.8, and 26.9 ± 1.3 μM, and the Hill coefficients were 0.9 ± 0.1, 1.1 ± 0.1, and 1.2 ± 0.1, respectively. Data are means ± SE (n = 6–14 cells). B: decay time constants of Kv1.5 K⁺, Rb⁺, and Cs⁺ currents in the presence of nifedipine. The reciprocal of the nifedipine-induced fast time constant of block (1/τ₂) at +40 mV for the different ions is plotted against the concentration of nifedipine. The solid line is the best fit to the data using the equation 1/τ₂ = kᵢ₋₁ × [D] + k₋₁ (see MATERIALS AND METHODS). The association rate constants (kᵢ₋₁) were 3.26 × 10⁶, 2.05 × 10⁶, and 1.33 × 10⁶ M⁻¹·s⁻¹ for K⁺, Rb⁺, and Cs⁺ currents, respectively, and the apparent dissociation rate constants (k₋₁) were 25.34, 33.88, and 36.16 s⁻¹ for K⁺, Rb⁺, and Cs⁺ currents, respectively. The equilibrium dissociation constants (Kᵢ) (k₋₁/kᵢ₋₁) for K⁺, Rb⁺, and Cs⁺ currents were 7.8, 16.5, and 27.2 μM, respectively. Data points are means ± SE (n = 3–14 cells).
Table 1. Time constants of Kv1.5 current decay in the presence of nifedipine

<table>
<thead>
<tr>
<th>Nifedipine Concentration, μM</th>
<th>K⁺, ms</th>
<th>Rb⁺, ms</th>
<th>Cs⁺, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>τ₁</td>
<td>τ₂</td>
<td>τ₁</td>
</tr>
<tr>
<td>2</td>
<td>31.3 ± 4.6</td>
<td></td>
<td>287 ± 21</td>
</tr>
<tr>
<td>5</td>
<td>221 ± 17</td>
<td>21.5 ± 1.6</td>
<td>298 ± 14</td>
</tr>
<tr>
<td>10</td>
<td>228 ± 14</td>
<td>17.2 ± 1.8</td>
<td>301 ± 24</td>
</tr>
<tr>
<td>20</td>
<td>209 ± 13</td>
<td>11.2 ± 1.8</td>
<td>302 ± 13</td>
</tr>
<tr>
<td>50</td>
<td>214 ± 15</td>
<td>5.5 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>2.8 ± 0.1</td>
<td></td>
<td>4.4 ± 0.6</td>
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</table>

Data are means ± SE; n = 3–13 cells. The Kv1.5 currents in the presence of nifedipine were fit by a double-exponential equation. The two time constants are shown with different concentrations of nifedipine. The time constants of the slow component (τ₂) were not significantly different compared with the rapid time constants of Kv1.5 current decays (τ₁) in the absence of nifedipine, which were fit with single-exponential equation (P > 0.05).

test whether or not Kv1.5 block by nifedipine was mediated within the pore region itself. The rationale was based on the preceding experiments, where it appeared that the presence of larger cations within the pore, like Cs⁺ and Rb⁺, reduced the potency of nifedipine binding compared with K⁺. The intracellular Cs⁺ concentration was kept constant at 130 mM, and the external ion concentration and species were changed to alter the direction of ion flow across the pore. A two-pulse protocol was used, with an initial 200-ms prepulse to index block of Kv1.5 outward Cs⁺ current at +80 mV by 20 μM nifedipine and a 200-ms test pulse immediately afterward to +30 mV to measure nifedipine block of current under different extracellular ion conditions (Fig. 3, A–D). Mean currents measured at the end of the prepulse and the test pulse are shown in Table 2. At the end of the 200-ms prepulse with an outward Cs⁺ current in all cases, there was no significant difference in the nifedipine block between extracellular Cs⁺ or K⁺ (Table 2). During the test pulse at +30 mV, as shown in Fig. 3, A, C, and D, an outward Cs⁺ current is present, and there is little difference in the action of nifedipine.

The essential observation is shown in Fig. 3B with 70 mM [K⁺]o, where the inward K⁺ current at +30 mV is significantly blocked by 20 μM nifedipine. The current is inward at this potential due to the higher permeability of the pore for K⁺ than Cs⁺. The current reduction is greater than that for any other ionic conditions (Table 2) and is the only condition where K⁺ is actually the permeating ion. In all other ionic conditions in these experiments, Cs⁺ is the permeating ion. These data support those in previous figures that indicated higher nifedipine potency with K⁺ in the bath and pipette rather than Cs⁺. These data extend that idea to show that it is the pore ion species that determines nifedipine potency. When K⁺ is the permeating ion, as in Fig. 3B during the test pulse, the blocking action of

Fig. 3. Nifedipine block is modulated by the ion species occupying the pore. The same twin-pulse voltage protocol was used in A–D in the presence of 130 mM intracellular Cs⁺ (Cs⁺i) concentration (A–D) and 70 mM extracellular Cs⁺ (Cs⁺o) concentration (A), 70 mM extracellular K⁺ (K⁺o) concentration (B), 5 mM Cs⁺o concentration (C), and 5 mM K⁺o concentration (D). Outward Cs⁺ current was elicited at +80 mV from a −80 mV holding potential during the first 200-ms pulse. During the second pulse, cells were depolarized to +30 mV for 200 ms. Current tracings are shown in the absence and presence of 20 μM nifedipine. Dashed lines in each panel denote the zero current level.
nifedipine is enhanced compared with the prepulse when Cs\(^+\) is the pore-permeating ion.

**Nifedipine action on Kv1.5 mutant channels.** The above experiments strongly support a pore-blocking action of nifedipine in Kv1.5. We wished to extend these experiments in a significant way by utilizing deep pore and outer pore mutants of Kv1.5. The first of these was a nonconducting mutant of Kv1.5, W472F. This single point mutant prevents ion permeation through the pore but otherwise gates normally, as indicated above each panel. Nonconducting mutant function is measured by recording gating currents as an index of channel gating and opening, as we (15, 29) have shown before.

In these experiments, the cation species on each side of the membrane was changed independently and the action of nifedipine tested on the return of gating current (\(I_{goff}\)) on repolarization. Channel block can be measured as a slowed return of gating charge on repolarization, because channel closing is slowed until the drug dissociates (32). Such effects are clearly seen in the data in Fig. 4. There is a prominent crossover of off-gating currents as they decay to the baseline in control and nifedipine treatments (e.g., Fig. 4, A–C) as a result of slowed closing of the channel in the presence of drug. With \(K^+_i\) or \(K^+_o\) (Fig. 4, A and D), the off-gating currents were greatly reduced and slowed in the presence of nifedipine, and the mean reduction of \(I_{goff}\) was significantly (\(P < 0.05\)) greater when extracellular \(K^+\) was present (72.9 ± 3.5\%) than when intracellular \(K^+\) was present (59.6 ± 4.3\%). When other cations were present in the bath or pipette solutions, less block of \(I_{goff}\) was observed. For intracellular and extracellular Rb\(^+\) concentrations, the reductions were 50.1 ± 3.5 and 57.9 ± 3.1\%, respectively, and for intracellular and extracellular Cs\(^+\) concentrations, the reductions were 41.1 ± 3.8 and 35.1 ± 6.4\%, respectively. These differences were also statistically significant by ANOVA (\(P < 0.001\)) among all external cations and between intracellular Cs\(^+\) and Rb\(^+\) concentrations or [K\(^+\)]. These data confirm the ionic current data described earlier: that nifedipine is a more potent blocker of Kv1.5 channels when [K\(^+\)]\(_o\) is present than when extracellular Rb\(^+\) or Cs\(^+\) are present. They also indicate that extracellular K\(^+\) allows greater block of Kv1.5 by nifedipine than intracellular K\(^+\).

The second mutant series that we have analyzed are changes to the outer mouth R487. This charged residue in Shaker channels is T449 (20) and is known to be a potent modulator of C-type inactivation as well as external TEA block in Shaker channels (14). A number of these mutants failed to express measurable current when transiently transfected into HEK cells, but two notable mutants were R487V and R487Y. Interestingly, these two mutants were those found by Lopez-Barneo et al. (20) to most strongly inhibit the development of C-type inactivation. The effects of nifedipine on Cs\(^+\), Rb\(^+\), and K\(^+\) currents through R487V-Kv1.5 expressed in HEK cells are shown in Fig. 5. At +40 mV, it is immediately apparent that the effect of nifedipine was reduced in this mutant compared with the wild-type channel, where the \(IC_{50}\) for inhibition of K\(^+\) current was 7.3 \(\mu\)M. Similarly, nifedipine was a less po-

**Table 2. Quantitation of pore ion modulation of nifedipine block**

<table>
<thead>
<tr>
<th>Time, ms</th>
<th>Normalized Current (I_{off}/I_{on})</th>
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<tr>
<td></td>
<td>70 mM [Cs(^+)](_i)</td>
</tr>
<tr>
<td>200</td>
<td>0.75 ± 0.03</td>
</tr>
<tr>
<td>400</td>
<td>0.68 ± 0.03</td>
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</table>

Data are means ± SE, \(n = 6–12\) cells. The normalized unblocked steady-state currents [current with nifedipine \(I_{off}/I_{on}\)] left after the prepulse at 200 ms and after the second pulse to +30 mV at 400 ms are from the data in Fig. 3. In all cases, the internal solution was 130 mM Cs\(^+\). The external solutions in each case are shown. Nifedipine (20 \(\mu\)M) was used in all experiments. [Cs\(^+\)]\(_o\), extracellular Cs\(^+\) concentration; [K\(^+\)]\(_o\), extracellular K\(^+\) concentration. *Significant difference (1-way ANOVA, \(P < 0.05\)) between 70 mM [K\(^+\)]\(_i\) and the other three external conditions.

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Fig. 4. Ion modulation of nifedipine (nif) block of gating current in Kv1.5-W427F. A–F: off-gating currents in control and in the presence of 100 \(\mu\)M nifedipine under different ionic conditions, as indicated above each panel. In each case, on-gating currents were unchanged in the presence of nifedipine (data not shown). NMG\(_i^+\) and NMG\(_i^+\), extracellular and intracellular N-methyl-D-glucamine concentration, respectively; K\(^+\) and K\(^-\), intracellular K\(^+\) concentration; Rb\(^+\) and Rb\(^-\), intracellular and extracellular Rb\(^+\) concentration, respectively; Cs\(^+\), intracellular Cs\(^+\) concentration.
Fig. 5. An external pore R487V mutant reduces nifedipine block. In all cases, pulses were from −80 mV to +40 mV for 400 ms. A: K⁺ currents; B: Rb⁺ currents; C: Cs⁺ currents. As indicated adjacent to the tracings in A–C, currents show block by different concentrations of nifedipine between 2 and 500 μM. In A–C, data are from the same cell. D: concentration-response curve for steady-state block of K⁺, Rb⁺, and Cs⁺ currents. Lines were fit to the data using the Hill equation. The IC₅₀ concentrations for K⁺, Rb⁺, and Cs⁺ currents blocked by nifedipine were 20.2 ± 0.5, 30.4 ± 0.6, and 50.7 ± 2.0 μM, and the Hill coefficients were 1.6 ± 0.1, 1.6 ± 0.04, and 1.6 ± 0.1, respectively. Data are means ± SE (n = 4–14 cells). The dotted lines represent the concentration-response relations for wild-type channels redrawn from Fig. 2.

Tent blocker of Rb⁺ and Cs⁺ currents than in the wild-type channel. Overall, the IC₅₀ values were increased to 20.2, 30.4, and 50.7 μM for K⁺, Rb⁺, and Cs⁺ currents, respectively. Along with the increased IC₅₀ values, the Hill slope was increased from that seen in the wild-type channel (Fig. 2) to ~1.6. The block of R487Y-Kv1.5 K⁺ currents by nifedipine was also tested. The IC₅₀ value was 21.6 ± 0.4 μM, and the Hill coefficient was 1.4 ± 0.03 (n = 6). These values are in close agreement with those obtained from the R487 mutant. It is known that these changes to the equivalent residue in Shaker channels result in quite local changes at the outer pore mouth and supports the idea that nifedipine effects on the channel depend on interactions in this outer mouth region of the channel.

Less potent action of nifedipine on Kv4.2 The observation that substitution of valine or tyrosine for arginine in the outer pore mouth of Kv1.5 reduced the IC₅₀ for nifedipine action significantly to ~20 μM, which prompted us to examine other Kv channels with a valine at this position. In a Shaker B T449V mutant channel, an IC₅₀ of ~30 μM was noted (1). The Shal channel Kv4.2 also has a valine at this equivalent position, and so we tested the ability of nifedipine to block this channel (Fig. 6). The rat Kv4.2 gene was stably expressed in HEK cells, and currents were recorded in the whole cell configuration as for Kv1.5. The Kv4.2 gene encodes an A-type rapidly inactivating outward K⁺ current, and block by nifedipine can be measured as peak current reduction (as nifedipine block is of rapid onset) or as an overall reduction in charge during the pulse. When currents were integrated over time, we obtained charge records, as shown in Fig. 6B. The concentration-response relations for peak current block and charge reduction by nifedipine are shown in Fig. 6C. Relations have similar IC₅₀ values of 32 and 29 μM for peak current and charge reduction, respectively, with Hill coefficients of 0.9. We applied the same single binding site model to the Kv4.2 data as was applied to results from Kv1.5 (Fig. 2). For Kv4.2 K⁺ currents, biexponential functions were required to fit the current decay both in the control and presence of nifedipine. As shown in Table 3, the slow component of Kv4.2 inactivation was not changed with the addition of nifedipine, suggesting that the drug did not change the inactivation rate. However, the time constant of the initial fast component was decreased depending on drug concentration, suggesting an overlap between drug block and the initial fast inactivation component.

If nifedipine block and inactivation are independent, the rate constants of the initial fast-decaying phase of Kv4.2 currents (1/τ_decay) in the presence of nifedipine should be a sum of the rate constants of channel inactivation (1/τ_inactivation) and of channel block (1/τ_block) (26) as follows:

\[ 1/\tau_{\text{decay}} = 1/\tau_{\text{block}} + 1/\tau_{\text{inactivation}} \]

Thus the value of 1/τ_block can be estimated by subtracting 1/τ_inactivation from 1/τ_decay. Assuming the inactivation process was not changed by nifedipine, we calculated 1/τ_block and plotted it as a function of nifedipine concentration in Fig. 6D. We fit the data with Eq. 2α and extracted kₐ (2.09 × 10⁶ M⁻¹·s⁻¹), k₋₁ (57.9 s⁻¹), and the Kₐ (27.7 μM) from the straight line fits. The Kₐ value was in good agreement with that obtained from the concentration-response relation (Fig. 6C) and suggested that the assumption of the model
was correct. These Kv4.2 results support the idea that a valine (or a tyrosine in Kv1.5) rather than an arginine at the outer pore mouth can reduce the affinity of Kv channels for nifedipine.

**Effects of \([K^+]_o\) on nifedipine block** Whole cell K+ currents during 5-s depolarizations to +40 mV are shown in Fig. 7A. The current trace in 5 mM \([K^+]_o\) shows a slow inactivation that is not complete at the end of a 5-s depolarizing step. The inactivation process can be fit using a double-exponential function (Eq. 3b in Data analysis). The resulting fast (\(\tau_2\)) and slow (\(\tau_1\)) time constants were 250 and 1,500 ms, respectively. Increasing \([K^+]_o\) to 135 mM slowed the inactivation process in parallel with an obvious reduction in current amplitude due to the decreased K+ driving force. After changing to 135 mM \([K^+]_o\), the time constants of inactivation were 400 (\(\tau_2\)) and 1,490 ms (\(\tau_1\)), respectively, by applying the same double-exponential function. The averaged time constants \(\tau_2\) and \(\tau_1\) in 5 and 135 mM \([K^+]_o\) were 265 ± 23 and 364 ± 32 ms (\(P < 0.05\)) and 1,750 ± 141 and 1,945 ± 145 ms, respectively. Data are means ± SE from three to four experiments. These data support the conclusion that C-type inactivation was slowed somewhat by elevation of \([K^+]_o\) and that this effect was caused by an effect on \(\tau_2\). Current traces from Fig. 7A were normalized to peak current and shown in Fig. 7B. This clearly showed that the C-type inactivation process was slower in 135 than 5 mM \([K^+]_o\). In contrast with the effects of external K+ on C-type inactivation, activation kinetics were not significantly altered by different external K+ concentrations (data not shown).

To investigate whether the time course and reduced C-type inactivation in high \([K^+]_o\) affected nifedipine block or not, 20 mM nifedipine was applied externally in the presence of 135 or 5 mM \([K^+]_o\). In both conditions, external application of 20 mM nifedipine not only markedly inhibited K+ current amplitudes but also apparently accelerated the decay rates of currents. These are shown clearly in Fig. 7A (inset) on an enlarged time scale. To clarify the relative rates of current decay, the current traces from Fig. 7A were normalized in Fig. 7B. In the absence of nifedipine, C-type inactivation in 135 mM \([K^+]_o\) was slower than that in 5 mM \([K^+]_o\). After exposure to 20 mM nifedipine, ~70% of steady-state current was blocked regardless of the \([K^+]_o\). This observation indicated that efficacy of current block by nifedipine was not significantly affected over this \([K^+]_o\) range. Besides effects on current amplitudes, 20 mM nifedipine obviously accelerated current decay in both 5 and 135 mM \([K^+]_o\), but the decay was slower in 135 mM \([K^+]_o\) than that in 5 mM \([K^+]_o\). This could be due to the existence of residual C-type inactivation whose time course was modulated (or
slowed) by the elevated external K$^+$ concentration. Alternatively, extracellular K$^+$ might directly influence nifedipine block. The nifedipine-sensitive currents shown in Fig. 7C were obtained by subtracting currents with nifedipine from currents in control. When normalized (as in Fig. 7D), nifedipine-sensitive currents showed identical rates of decay. This was consistently observed in three other experiments with 20 mM nifedipine and in experiments with lower and higher nifedipine concentrations. Nifedipine-sensitive currents were well fit by a single-exponential function (Eq. 3a), and the resulting time constants were 1,259 and 1,150 ms. Therefore, the different decay rates of currents after exposure to 20 mM nifedipine shown in Fig. 7B resulted from different C-type inactivation kinetics caused by different K$^+_o$ concentrations rather than an alteration in the kinetics of nifedipine block.

The study was extended to test higher concentrations of K$^+_o$, as shown in Fig. 8. The aim was to test a series of [K$^+$]$_o$ in each cell, and this was done by using shorter 400-ms voltage-clamp pulses and changing the external [K$^+$] while cells were exposed to a single nifedipine concentration. The normalized current records in Fig. 8A and the bar graph in Fig. 8B summarize the amount of block caused by 10 mM nifedipine at each [K$^+$]$_o$ and show that there was little effect of changing [K$^+$]$_o$ over this range on the amount of block induced by nifedipine. All these results are consistent with the idea that nifedipine block is a distinct process from C-type inactivation. This is further supported by our experiments on recovery from C-type inactivation.

Effects of nifedipine on recovery from C-type inactivation. To study recovery from C-type inactivation in the absence and presence of nifedipine, a double-pulse voltage protocol was used. The purpose of the first pulse (prepulse) was to predominantly activate (during short prepulses) or to activate and then inactivate the channel (during long prepulses). After a variable inter-
pulse duration, the second pulse (test pulse) was applied to test how many channels had recovered from the inactivated state induced by the prepulse. The fractional recovery was therefore defined as follows: fractional recovery = $I_{\text{peak2}}/I_{\text{peak1}}$, where $I_{\text{peak1}}$ and $I_{\text{peak2}}$ represent the peak currents elicited by the prepulse and test pulse depolarizations, respectively. The current traces shown in Fig. 9 were elicited by double-pulse protocols with long and short pulse durations over a range of interpulse intervals. All pulses were to +40 mV. The holding potential and the potential during the interpulse intervals was −80 mV. The interval between each trace was 30 s to ensure that no inactivation accumulated between each cycle of the protocol.

In Fig. 9, A and B, the currents are in response to a long 5-s prepulse with interpulse intervals of variable duration from 180 ms to 3 s in increments of 400 ms. In the absence of nifedipine (Fig. 9A), more than 50% of the current inactivated during the 5-s prepulse at +40 mV. After a brief interval, peak current amplitudes recorded during test pulses ($I_{\text{peak2}}$) were smaller than those of the first prepulse ($I_{\text{peak1}}$). $I_{\text{peak2}}$ recovered slowly with increasing interpulse intervals and reached only 85% of $I_{\text{peak1}}$ after an interpulse interval of 3 s. When 20 μM nifedipine was applied externally (Fig. 9B), both peak and steady-state currents at +40 mV were rapidly inhibited during the prepulse.

In the absence and presence of 20 μM nifedipine, averaged fractional recovery was plotted against the interpulse intervals (Fig. 9E). Control data points were well fit by a single-exponential function, with a mean recovery rate of 1.8 ± 0.1 s (means ± SE, n = 8), which reflected the slow recovery from C-type inactivation. In the presence of 20 μM nifedipine, data points were fit by a double-exponential equation, and the mean recovery rates were 2.8 ± 0.3 s ($\tau_1$) and 0.33 ± 0.02 s ($\tau_2$), means ± SE, n = 7). Although the slow time constants ($\tau_1$) in control and nifedipine are not in exact agreement, the data suggest that, in the presence of nifedipine, recovery after long depolarizations comprised two processes. One of these was the slow recovery from C-type inactivation ($\tau_1$) seen in the control and also still present, although somewhat slower, with nifedipine. The second additional fast recovery was likely to be recovery of noninactivated channels from open channel nifedipine block ($\tau_2$) during the repolarization (interpulse) interval. The initial rapid phase of recovery from block was small, and most of the recovery in the presence of nifedipine consisted of a slow recovery from C-type inactivation with a similar amplitude and time course to that observed in control. This clearly indicated that most of the channels rapidly blocked by nifedipine had subsequently inactivated during the prepulse (Fig. 9B), and these channels recovered slowly. The fewer channels in the nifedipine-blocked but noninactivated state were able to recover rapidly on repolarization.

The separate nature of recovery from nifedipine block and C-type inactivation was further illustrated by applying a short double-pulse protocol, as indicated in Fig. 9C. Here, currents were elicited by pairs of 60-ms depolarizing pulses to +40 mV with a variable interpulse interval from 120 to 1,920 ms in increments of 200 ms. The prepulse activated Kv1.5 channels and allowed nifedipine block while letting as few channels as possible become C-type inactivated. The data in Fig. 9C show that, in the absence of nifedipine, currents activated at +40 mV minimally inactivated during the prepulse and were fully recovered after an interpulse interval of 120 ms. In the presence of 20 μM nifedipine, current was significantly blocked during the 60-ms prepulse. Currents only partially recovered during test pulses after an interval of 120 ms. Mean data for fractional recovery during short double-pulse experiments are shown in Fig. 9F. The data points clearly
showed that, in the absence of nifedipine, little inactivation occurred during the prepulse, and data could be fit using a straight line. In the presence of 20 μM nifedipine, data were fit by a single-exponential equation to give a recovery rate of peak current from nifedipine block of 316 ± 19 ms. Since little inactivation occurred over this duration, this time constant reflected the recovery from nifedipine block and was consistent with the faster of the two rates obtained from Fig. 9E (332 ms). It seems, then, that whether inactivation was permitted [as during the long prepulse (Fig. 9, A and B)] or not given time to develop during the short prepulse (Fig. 9, C and D), nifedipine block and recovery occurred at the same rates. This strongly suggests that nifedipine block of Kv1.5 channels and C-type inactivation are independent processes.

DISCUSSION

Influence of permeating ions on Kv1.5 channel block by nifedipine. It has been reported before that permeating ions can affect the efficacy of channel block by charged drugs like TEA (3, 16). In cloned Kv2.1 channels, external application of 30 mM TEA can block K+ currents by 87%, whereas the same external concentration of TEA has no effects on Na+ currents through the same channel (16). Here, we have shown that, like TEA, nifedipine also has a different potency for block of K+, Rb+, and Cs+ currents through another Kv channel, Kv1.5 (Figs. 1 and 2). Because TEA block is coordinated by all four K+ channel subunits, although Na+ ions are smaller than K+, it seems most likely that binding of K+ rather than Na+ within the permeation pathway allows the formation of the TEA or nifedipine binding sites. Alternatively, it is possible that nifedipine binds with the same affinity to channels that pass both K+ and Cs+ and that the larger cation can more easily escape the blocker and pass through the channels. The pore configuration that would allow this possibility is uncertain, and the data in Fig. 3B showed increasing block of the inward current during the switch from outward Cs+ to inward K+ current. This suggests that the nifedipine binding affinity was different in the presence of different ions.

When K+, Rb+, and Cs+ ions permeate through Kv1.5 channels, their different sizes induce unique conformational changes in the structure of the pore that might affect the binding site of nifedipine itself or the ability of nifedipine to access such a site. In the latter case, the binding site(s) of permeating ions within the channel pore could be close to the binding site of nifedipine, and the larger cations might affect access of nifedipine to its binding site by steric hindrance. We (32) had previously reported that nifedipine blocked Kv1.5 with an apparent distance of 12–16% of the electric field from the outside. It was shown in Fig. 1 that there was no obvious change in the voltage dependence of block with the three different cations, which suggests that the site of action of nifedipine in the outer pore mouth had not significantly moved. A single-binding site model was still appropriate to describe the kinetics of nifedipine block by the three different cations. The slopes of the concentration-response relations had Hill coefficients close to 1.0 (Fig. 2), and the concentration dependence of the acceleration of current decay by the drug produced values for on- and off-drug binding rates and $K_b$ that closely fitted a single site model. This also validated an open-channel block mechanism by nifedipine in the presence of the three different cations.

Site of nifedipine action on Kv1.5. Nifedipine action on Kv1.5 appears to be closely tied to the ion conduction pathway. The drug does not block closed channels but rapidly blocks open channels causing a rapid current decay superimposed on slower inactivation when activation is sufficiently rapid to allow this to be seen (Fig. 1). The influence of different permeating cations on the rate and potency of the block of ionic and gating currents is further evidence for effects within the open pore. In conducting channels, when K+ was the permeating cation (as in Fig. 3B at +30 mV), block was more extensive than when Cs+ was the permeating cation. Overall, the data showed that the permeating cation was the most important determinant of block (Table 1). When ion permeation was prevented in the W472F nonconducting mutant and gating currents were measured (Fig. 4), it was shown that extracellular K+ allows greater block of Kv1.5 by nifedipine than other extracellular cations. Interestingly, though, in these experiments, addition of only intracellular K+ or Rb+ was able to coordinate a relatively potent block of off-gating current by nifedipine. This suggested that even intracellular ions were able to have long range effects on overall pore conformation and affect the formation of the nifedipine binding site in the outer channel mouth without being present there.

Pore mutants and their effects. Two outer pore mutants of Kv1.5, R487V and R487Y, affected the concentration dependence of nifedipine block of the channel. Low concentrations of nifedipine were much less effective on these mutant channels regardless of the ionic species, but there was a steepening of the concentration-response relationships so that at higher concentrations nifedipine apparently blocked the channel to the same degree as the wild-type channels, and the overall efficacy was unchanged (Fig. 5). The ion-dependent shift in the IC$_{50}$ of the concentration-response relations was maintained but reduced in relative terms, so that whereas Cs+ was 4 times less potent in the wild-type channels, it was 2.5 times less potent in the R487V mutant. This result suggested that the ion effects on nifedipine potency could be separated to a large degree from effects of outer pore mutations on nifedipine binding to Kv1.5 and that ion species effects were not caused by interaction directly at the nifedipine binding site. This argues against a common site for nifedipine block and ion modulation of block in Kv1.5.

The steepening of the concentration-response relations in the R487V mutant increased the Hill coefficients from $-1$ to $-1.6$ and suggested that the muta-
tion was able to significantly alter the mechanism by which nifedipine binds to Kv1.5. It is possible that substitution of the positively charged arginine by the hydrophilic arginine or the planar ring of tyrosine facilitates additional molecules of nifedipine binding to the channel, perhaps forming another binding site for nifedipine. Overall affinity is reduced as evidenced by the decreased potency, but once a raised nifedipine concentration is present at the outer pore mouth, it is possible that the binding of a second nifedipine is facilitated, and channel block is enhanced. The valine substitution in Shaker channels results in a similar potency to that in Kv1.5 (1), and a similarly low potency was observed in our experiments in Kv4.2 channels, which have a valine at the equivalent site to R487 in Kv1.5 (Fig. 6). In this situation, though, the presence of valine per se does not confer a cooperative action of nifedipine, and the concentration-response relation had a Hill coefficient close to 1.0.

Nifedipine block and C-type inactivation. The principal action of nifedipine on Kv1.5 was to accelerate the rate of current decay once the channel-opening rate significantly exceeded blocking rate. The possibility existed, because permeant cations modulated the degree of nifedipine block, that the action of nifedipine was in some way related to an acceleration of C-type inactivation. This is based on the knowledge that permeant cations, and especially extracellular cation concentrations, are effective modulators of the rate of C-type inactivation (20) and the recovery from inactivation. In addition, the residue R487 in Kv1.5 is analogous to T449 in Shaker channels in that both are important determinants of the inactivation rate. In Kv1.5, though, the role for this residue is less critical (8), and, as discussed below, quite large changes in [K+]o have less pronounced effects on the inactivation rate than in Shaker channels. Modulation of C-type inactivation by channel blockers has been extensively studied by internal or external applications of TEA, where external TEA competes for C-type inactivation (6, 11). Interactions between C-type inactivation and channel blockers is manifested in many ways. In Kv1.3 channels in T lymphocytes, slowed C-type inactivation via exposure to 160 mM [K+]o greatly diminished the potency of CP-339,818, a channel blocker of Kv1.3 (22), suggesting that CP-339,818 preferentially inactivated the channel, perhaps forming another binding site for nifedipine. Overall, the experiments indicate that the outer pore mouth block is mediated at a nifedipine binding site that is relatively unaffected by the processes involved in the onset and development of C-type inactivation in this channel.

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