

Dihydropyridine Action on Voltage-dependent Potassium Channels Expressed in *Xenopus* Oocytes

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ABSTRACT Dihydropyridines (DHPs) are well known for their effects on L-type voltage-dependent Ca^{2+} channels. However, these drugs also affect other voltage-dependent ion channels, including *Shaker* K^+ channels. We examined the effects of DHPs on the *Shaker* K^+ channels expressed in *Xenopus* oocytes. Intracellular applications of DHPs quickly and reversibly induced apparent inactivation in the *Shaker* K^+ mutant channels with disrupted N- and C-type inactivation. We found that DHPs interact with the open state of the channel as evidenced by the decreased mean open time. The DHPs effects are voltage-dependent, becoming more effective with hyperpolarization. A model which involves binding of two DHP molecules to the channel is consistent with the results obtained in our experiments.

KEY WORDS: potassium channels • dihydropyridines

INTRODUCTION

Dihydropyridines (DHPs)¹ with a 1,4 dihydropyridine ring and a phenyl ring are clinically used to treat a variety of cardiovascular disorders, primarily acting as vasodilators. Effects of the DHPs on L-type Ca^{2+} channels are especially interesting in that the DHPs can be either agonists or antagonists (Reuter et al., 1988; Hess, 1990). These drugs appear to act allosterically to modify the channel function by promoting different modes of gating (Hess, 1990). They have greater affinities for the inactivated state of the channel, which is favored by depolarization (Hess, 1990).

Although DHPs have profound effects on L-type Ca^{2+} channels, it was shown that these compounds also affected voltage-dependent Na^+ channels (Yatani et al., 1988) and voltage-dependent K^+ channels (Jacobs and DeCoursey, 1990; Gotoh et al., 1991). Using the whole cell voltage-clamp method, Jacobs and DeCoursey (1990) tested a series of clinically relevant agents, including nifedipine, on the K^+ currents in alveolar epithelial cells and found that nifedipine produced apparent inactivation. They suggested that nifedipine, at least in part, works by acting as an open channel blocker. Gotoh et al. (1991) studied the effect of nicardipine on the K^+ channel current in rabbit heart cells. They found that nicardipine accelerated the time course of the outward K^+ current decline with the 50% inhibitory concentration of 630 nM. This is fourfold higher than that for the Ca^{2+} current in the same cells (160 nM).

The structural elements involved in the DHP binding to the Ca^{2+} channels are not thoroughly understood. Biochemical experiments (Nakayama et al., 1991; Regulla et al., 1991) using antibody mapping indicated that DHPs may bind to the amino acid residues in the S6 segment of the α_1 subunit of the Ca^{2+} channel. Some investigators (Glossmann et al., 1984; Triggle et al., 1989) have suggested that Ca^{2+} channels may have two DHP binding sites, a high-affinity site (nM range) and a low-affinity site (μM range). A mutagenesis study using chimeric channels based on two distinct Ca^{2+} channels with different DHP affinities shows that the S5, Pore, and S6 segments may be important in determining the DHP sensitivity (Grabner et al., 1996). A molecular simulation study showing how a DHP may bind to the Ca^{2+} channel pore with Ca^{2+} ions bound suggests that DHPs occlude the channel pore (Zhorov and Ananthanarayanan, 1996).

We investigated the effects of DHPs on the *Shaker* K^+ channels expressed in *Xenopus* oocytes. Native cells may express more than one class of K^+ channels and the interpretations of the results of the drug action are usually not straightforward. Heterologous expression of a single class of channel can largely overcome this problem. Since the biophysical gating properties of the *Shaker* channel have been studied extensively (Bezanilla et al., 1994; Hoshi et al., 1994; Stefani et al., 1994; Zagotta et al., 1994*a,b*), any alterations in the channel gating can be more easily detected and studied. Furthermore, molecular manipulations of the *Shaker* channel affecting some of the gating transitions, such as inactivation rates, have been described (Hoshi et al., 1990, 1991; Zagotta et al., 1990; Lopez-Barneo et al., 1993), and they can be used to probe the interactions of the channel with DHPs. The results obtained in this study indicate that DHPs interact with the open state of the

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¹Abbreviations used in this paper: DHP, dihydropyridine; NMG, *n*-methyl-D-glucamine; TEA, tetraethylammonium.

Shaker channel inducing apparent inactivation although the drugs do not act as simple open-channel blockers. The results also show that the overall DHP efficacy can be modulated by a single amino acid mutation in the S6 segment.

MATERIALS AND METHODS

Channel Expression

Wild-type and mutant *Shaker* K⁺ channels were expressed in *Xenopus* oocytes essentially as described previously (Hoshi et al., 1990). The following channels were used in this study. *ShD* (Timpe et al., 1988) is characterized by intact N-type inactivation and C-type inactivation. *ShBΔ6-46* contains a 41-residue deletion in the amino terminus and lacks N-type inactivation (Hoshi et al., 1990). In addition to the amino terminus deletion, *ShBΔ6-46* T449V contains a single amino acid substitution at position 449 (using the *ShB* numbering) from T to V. The *ShBΔ6-46* T449V channel has disrupted N-type inactivation and drastically slowed or disrupted C-type inactivation (Lopez-Barneo et al., 1993). *ShBΔ6-46* T449K has a point mutation at position 449 from T to K to accelerate C-type inactivation to a more experimentally manageable range (Lopez-Barneo et al., 1993). *ShBΔ6-46* T449V: A463I has an additional point mutation at position 463 that has been shown to be involved in C-type inactivation (Hoshi et al., 1991). The RNAs were transcribed using T7 RNA polymerase (Ambion, Austin, TX) and injected into the oocytes (40 nl/cell). Recordings were typically made 1 to 14 d after injection.

Electrophysiological Recording

In most experiments, the single channel and macroscopic patch currents were recorded using excised configurations of the patch-clamp technique (Hamill et al., 1981; Methfessel et al., 1986) with an AxoPatch 200A amplifier (Axon Instruments, Foster City, CA). The macroscopic patch currents were low-pass filtered through an eight-pole Bessel filter unit with 2 kHz corner frequency (Frequency Devices, Haverhill, MA) and digitized at 10 kHz using an ITC16 computer interface (Instrutech, Great Neck, NY). The whole oocyte currents were recorded using a Warner OC-725B (Warner, Hamden, CT) two-electrode voltage clamp amplifier. The electrodes filled with 3 M KCl had a typical resistance of less than 0.5 MΩ. The data were collected and analyzed using Pulse/PulseFit (HEKA, Lambrecht, Germany), IGOR PRO (Wavemetrics, Lake Oswego, OR), TAC (SKALAR, Seattle, WA), and DataDesk (DataDescriptions, Ithaca, NY) programs running on Apple Macintosh computers. Unless otherwise indicated, the linear capacitative and leak currents have been subtracted from the macroscopic currents presented using a modified P/n protocol. The single channel data were idealized using the half amplitude threshold method. For the analysis of the single channel dwell times, additional exponential components were included only if the probability of requiring one additional component was >0.95 using the likelihood ratio test (Hoshi et al., 1994). Numerical simulations of the ionic currents predicted by the kinetic schemes were performed using the Q-matrix approach (Colquhoun and Hawkes, 1995) implemented in IGOR. The parameters of the schemes were optimized by minimizing the chi-squared value using the downhill simplex algorithm (Press et al., 1994) implemented in IGOR. When appropriate, the data values are presented as mean ± standard deviation. The error bars are not shown when smaller than the symbol size. All experiments were performed at room temperature (20–24°C).

Solutions

The intracellular solution typically contained (in mM): 140 KCl, 11 EGTA, 10 HEPES, pH 7.2 (titrated with *n*-methyl-D-glucamine [NMG]). The extracellular solution typically contained (in mM): 140 NaCl, 2 MgCl₂, 2 KCl, 10 HEPES (NMG), pH 7.2. The high K⁺ extracellular solution contained (in mM): 140 KCl, 2 MgCl₂, 10 HEPES (NMG), pH 7.2. Other solutions used are indicated in the legends.

Nifedipine (Sigma Chemical Co., St. Louis, MO), Bay K 8644 (RBI, Natick, MA), and nimodipine (Sigma) were dissolved in 100% ethanol (10 mM). Nicardipine (Sigma) was dissolved in 100% methanol (5 mM). The stock solutions were kept in the dark at <4°C. The drug solutions were prepared fresh from these stock solutions and vortexed immediately before each use.

RESULTS

The *ShBΔ6-46* T449V channel contains a deletion in the amino terminus to disrupt N-type inactivation (Hoshi et al., 1990). In addition, threonine at position 449 (in *ShB* numbering) in the P-segment is mutated to valine to disrupt or drastically slow C-type inactivation (Lopez-Barneo et al., 1993). Effects of DHPs were primarily assayed using this *ShBΔ6-46* T449V channel since the gating transitions are simplified with the N- and C-type inactivation mechanisms disrupted. Because internal Mg²⁺ ions are known to induce voltage-dependent block of currents through *Shaker*-like potassium channels (Ludewig et al., 1993), all data presented were recorded in the absence of internal Mg²⁺ ions to exclude interference with divalent block in interpreting the DHP effects.

Nifedipine Induces Apparent Inactivation

Application of nifedipine to the intracellular side induced a time-dependent decline in the ionic currents through the *ShBΔ6-46* T449V channels. Fig. 1 A shows the effects of intracellular nifedipine (50 μM) on the *ShBΔ6-46* T449V K⁺ currents elicited in response to depolarizing voltage pulses. The amplitudes of the control *ShBΔ6-46* T449V currents did not decline appreciably with maintained depolarization. Most of the reduction in the control current amplitude observed with maintained depolarization is attributable to accumulation of K⁺ ions in the extracellular space immediately adjacent to the channels as judged by the changes in the reversal potential of the tail currents with time (data not shown). In the presence of nifedipine, the currents underwent marked apparent inactivation at the positive voltages where the activation process is fast, suggesting that nifedipine exerts its action after the channel opens. The current-voltage (I-V) curves indicate that the reduction of the current amplitude by nifedipine is apparently voltage dependent (Fig. 1 B).

The effect of nifedipine application to the intracellular side to induce apparent inactivation had a very fast onset, much faster than the limit of the manual bath

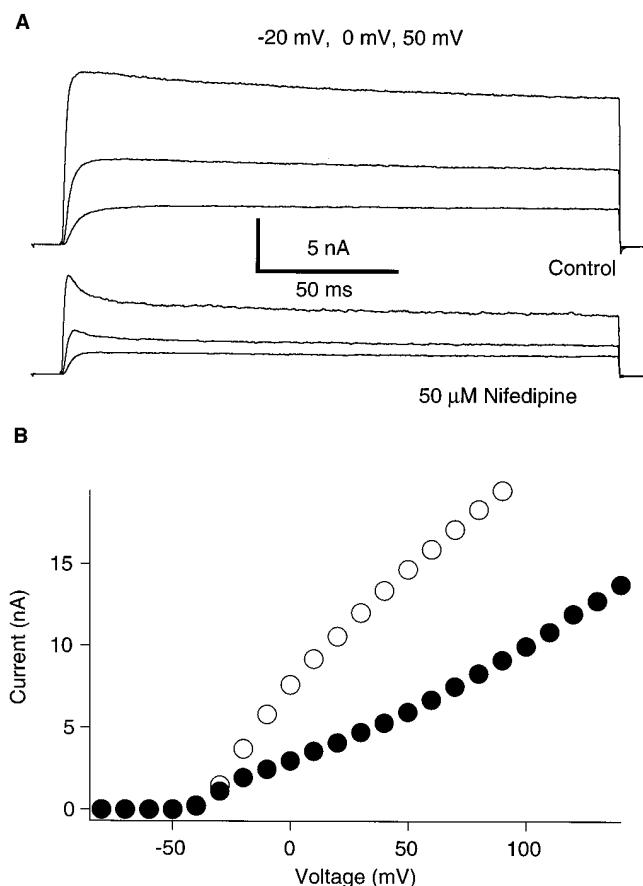


FIGURE 1. Nifedipine block of K^+ currents through *ShBΔ6-46* T449V channels. (A) Current traces obtained from the inside-out patch in response to voltage steps from -100 mV to -20 mV, 0 mV, and $+50$ mV with (bottom) and without (top) nifedipine ($50 \mu\text{M}$) in the internal solution are shown. (B) Current amplitudes measured at the end of 200-ms pulses with (closed circles) and without (open circles) nifedipine ($50 \mu\text{M}$) in the internal solution.

perfusion system employed. The effect was readily and fully reversible. Applications of nifedipine to the extracellular side were less effective than those to the intracellular side (Fig. 2 A). Extracellular applications of nifedipine ($100 \mu\text{M}$) often induced a time-independent decrease in the current amplitude (inset traces in Fig. 2 A). These observations indicate that nifedipine works preferentially from the intracellular side. Considering the non-polar nature of nifedipine, the effects of extracellular application were probably mediated by its movement through the cell membrane.

DHPs are light-sensitive and the effects on voltage-dependent Ca^{2+} channels can be markedly diminished by UV-light (Meyer et al., 1984). We examined whether the nifedipine effects on the K^+ channels could be diminished by UV-light. We found that UV-treated nifedipine (254 nm , 18 W , for 90 min at 1 cm) was much less effective in producing the apparent inactivation of

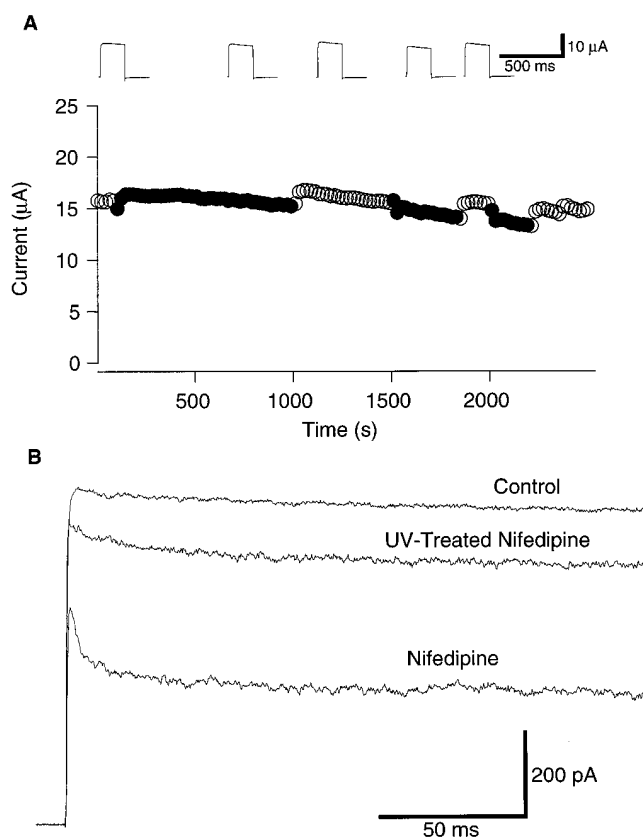


FIGURE 2. (A) Nifedipine applied externally is less effective in blocking the ionic currents through the *ShBΔ6-46* T449V channel. Peak amplitudes of the currents measured using the two-electrode voltage clamp during voltage pulses from -100 mV to $+50$ mV are plotted (filled circles: data measured during nifedipine ($100 \mu\text{M}$) application, open circles: wash out of drug). Representative current sweeps recorded at the end of the corresponding segment are shown in inset. (B) Nifedipine, exposed to ultraviolet light, is less effective in blocking the *ShBΔ6-46* T449V current. Representative inside-out macro-patch currents in response to pulses from -100 mV to $+50$ mV. A half of the nifedipine solution ($50 \mu\text{M}$) was treated with UV light (254 nm 18W for 90 min at 1 cm) and the efficacy of the UV-treated nifedipine was compared with the other half of the nifedipine solution, which did not receive UV treatment.

the *ShBΔ6-46* T449V currents (Fig. 2 B). The vehicles (ethanol, methanol) did not induce the inactivation at the concentrations used.

Other Dihydropyridines Are Also Effective

In addition to nifedipine, other DHPs are also effective in inducing apparent inactivation in the *Shaker* channels. Fig. 3 compares the intracellular applications of nimodipine, nicardipine, and BAY K 8644 (+, -) on the *ShBΔ6-46* T449V currents elicited in response to the voltage pulses to $+50$ mV. As with nifedipine, these DHPs induced apparent inactivation in a reversible manner when applied to the intracellular side. Extra-

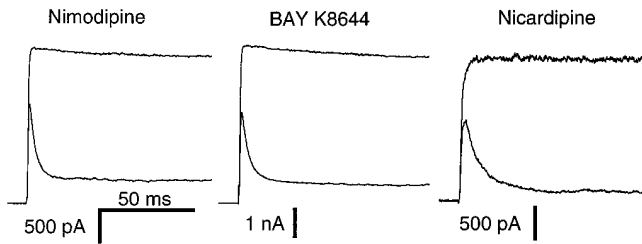


FIGURE 3. Block of the *ShB*Δ6-46 T449V macroscopic currents by different DHPs. Currents were recorded in the inside-out configuration in response to depolarizing voltage steps to 0 mV. Indicated DHPs were applied to the internal side. The concentration was 100 μM for all DHPs.

cellular applications of these drugs were also much less effective (data not shown).

Concentration Dependence of DHP Block

The currents recorded at +50 mV in the presence of various concentrations of nifedipine are shown in Fig. 4 A. Fig. 4 C–F show the concentration dependence of the block of the macroscopic currents at +50 mV by nifedipine and nimodipine. The effect of nifedipine at +50 mV became noticeable at 3 μM with the half-maximal reduction in the steady-state current occurring at ~30 μM (Fig. 4 C). For nimodipine, the half-maximal reduction was observed at a slightly lower concentration (Fig. 4 E). With greater concentrations, the apparent inactivation time course became faster and the steady-state current became progressively smaller. These results are again consistent with the drugs exerting the action after the channel opens. The fractions of the currents blocked at the end of the sweep are shown in Fig. 4 C (nifedipine) and E (nimodipine). The time constants of the current decline are shown in Fig. 4 D (nifedipine) and F (nimodipine). These plots illustrate a slight difference in the efficacies of nifedipine and nimodipine. The Hill plot transformations of the block data (Fig. 4 B) indicate that the concentration dependence of both nifedipine and nimodipine block has the Hill coefficient of 1.5, consistent with more than one DHP molecule binding to the channel.

Nifedipine Decreases the Mean Open Time

If nifedipine interacts with the open state of the channel, the mean open time should decrease in a concentration-dependent manner. To examine the effect of nifedipine on the single channel level, a mutant *Shaker* channel, *ShB*Δ6-46 T449V:A463I, which contains a single amino acid substitution at position 463 (Hoshi et al., 1991) in the *ShB*Δ6-46 T449V background was used. The A463I mutation increases the single channel amplitude by 50%, and it increases the mean open time by 20–30-

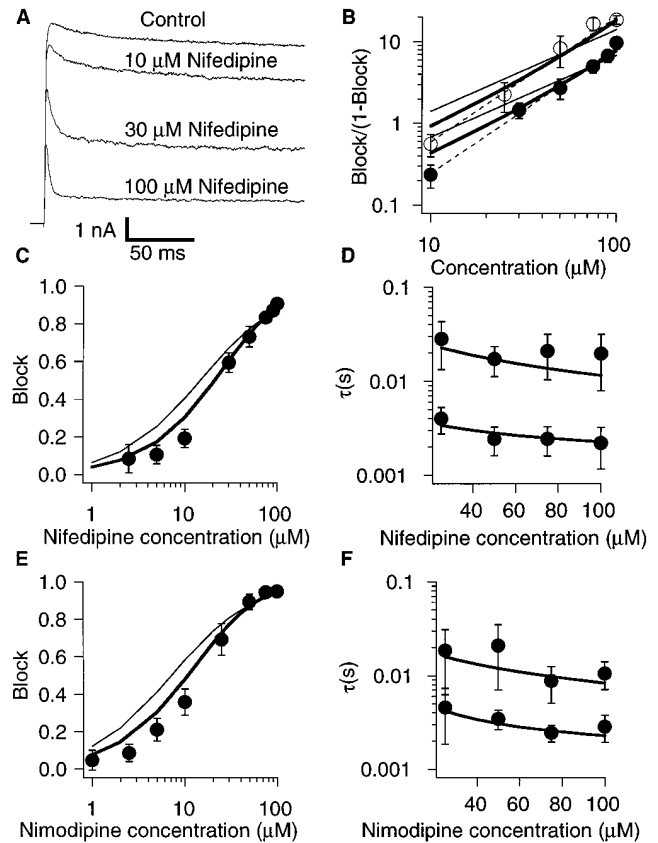


FIGURE 4. Concentration dependence of DHP block of the macroscopic currents through the *ShB*Δ6-46 T449V channels. (A) Current traces obtained in response to voltage steps to +50 mV with the indicated concentrations of nifedipine in the internal solution. (B) Hill plot of the concentration dependence of block of *ShB*Δ6-46 T449V channel by nimodipine (open circles) and nifedipine (closed circles). Block is given by $I_{drug}/I_{control}$. Dashed lines are the least square fits of the data with Hill equation. Both nifedipine and nimodipine fits give a Hill coefficient value of 1.5. (C) Relative reduction of the steady state current measured at the end of a 200-ms voltage pulse to +50 mV by different concentrations of nifedipine. Values on the vertical axis were calculated as $1 - I_{nifedipine}/I_{control}$. Data points represent mean \pm standard deviation of six experiments. (D) Time constants of the current decline induced by nifedipine block. The currents elicited by 200-ms voltage pulses to +50 mV in the presence of nifedipine at the concentrations indicated were fitted with a sum of two exponentials. (E) Relative reduction of the steady-state current by nimodipine. The data were collected and analyzed as in C. (F) Time constants of the current decline in the presence of nimodipine (averages of four to seven experiments). The data were collected and analyzed as in D. In B–F, smooth curves show least square fits of the data obtained from the simulated currents as described in DISCUSSION (thin line, SCHEME II, thick line, SCHEME V).

fold. Thus, this channel is well suited to assay the DHP effects. Representative openings of a single *ShB*Δ6-46 T449V:A463I channel recorded at 0 mV are shown in Fig. 5 A. Application of nifedipine decreased the mean open time in a concentration-dependent manner (Fig.

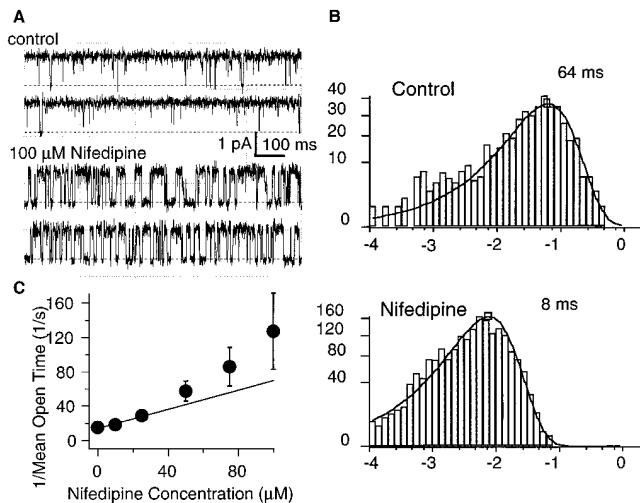


FIGURE 5. Nifedipine decreases the mean open time in *ShBΔ6-46 T449V:A463I*. (A) Representative openings in the absence (*upper traces*) and in presence of 100 μM nifedipine (*lower traces*) in the internal solution. Inside-out configuration at 0 mV. Closed state is indicated by dashed line. The data were filtered at 1.8 kHz. The patch contained one functional channel. (B) Open time histograms with and without 100 μM nifedipine. Data are shown in square root scaling on vertical axis (Sigworth and Sine, 1987). Single exponential fits are shown superimposed. (C) Reciprocals of the mean open times at several concentrations of nifedipine. Each data point represents the mean \pm standard deviation of four to six experiments. Solid line shows linear least square fit of first three points.

5, B and C). The reduction of mean open time is consistent with nifedipine interacting with the open state of the channel. The amplitude of the main conductance state of the channel was not markedly affected by nifedipine application. Although we observed the substates more frequently in the presence of nifedipine, we did not systematically investigate this issue.

The closed durations recorded from the data shown in Fig. 5 are compared in Fig. 6. In the absence of the drug, the closed durations were described by a sum of at least three exponentials. The shortest exponential component with a time constant value of 200–300 μs reflects the short burst closures as observed for the *ShBΔ6-46* channel, C_f (Hoshi et al., 1994). The second component with a time constant value of 2–4 ms reflects the intermediate closed state, C_i (Hoshi et al., 1994). In addition to these closed duration components, similar to those already documented for the *ShBΔ6-46* (Hoshi et al., 1994), the closed durations from the *ShBΔ6-46 T449V:A463I* channel also showed an additional component with a time constant value of 50–200 ms with a small fractional amplitude (3% in the data shown). Nifedipine (100 μM) did not introduce an additional closed duration component in a statistically significant

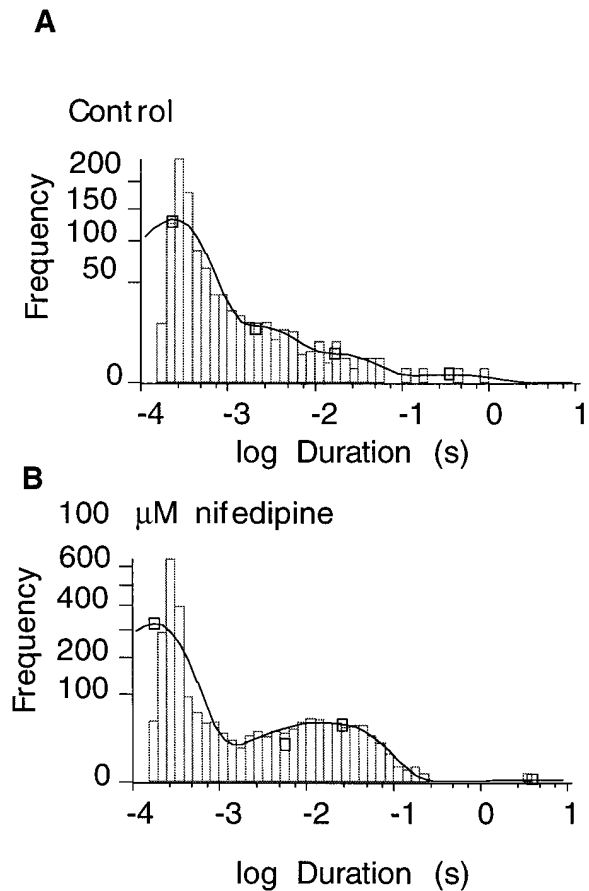


FIGURE 6. Nifedipine effects on the closed time distribution of *ShBΔ6-46 T449V:A463I*. Closed time distributions in the absence (A) and in the presence (B) of 100 μM nifedipine. Data are shown in square root scaling on vertical axis. Lines shown represent four exponential fits of the distributions. Under control conditions the time constants (fractional amplitudes) were 0.2 ms (0.871), 2.1 ms (0.097), 16.8 ms (0.029), and 352 ms (0.003). For nifedipine, the three time constants (fractional amplitudes) were 0.2 ms (0.84), 5.7 ms (0.048), and 26 ms (0.109).

way but noticeably increased the relative amplitude of the third component (from 3% to 11% in Fig. 6). It is possible that nifedipine did induce an extra component, but the new time constant was too similar to one of the existing time constants to be detected. Similar results were obtained from two other single-channel patches.

Interaction between the Nifedipine Block and N- and C-type Inactivation

Since nifedipine induces apparent inactivation, we studied whether nifedipine interacts with N-type or C-type inactivation. We first examined the possible interaction between nifedipine and the N-type inactivation mechanism by using the *ShD* channel (Timpe et al., 1988),

which exhibits fast N-type inactivation. As described earlier (Choi et al., 1991), in the presence of internal tetraethylammonium (TEA) (2 mM), the current decline is slower since the TEA-bound *ShD* channels cannot undergo N-type inactivation, and a cross-over of the scaled current time courses was observed (Fig. 7 A). In the absence of TEA, the time course of the current decline was well fitted by a single exponential with a time constant of 9 ms whereas with TEA, a sum of two exponentials with time constant values of 2 and 26 ms was required to fit the current decline. In contrast, decline of the *ShD* current in the presence of nifedipine was not slower than the control current and the scaled currents superimposed well (Fig. 7 B). Because the apparent on and off rates of nifedipine are slower than those of TEA (see Fig. 1), the results do not necessarily show that N-type inactivation and nifedipine do not compete.

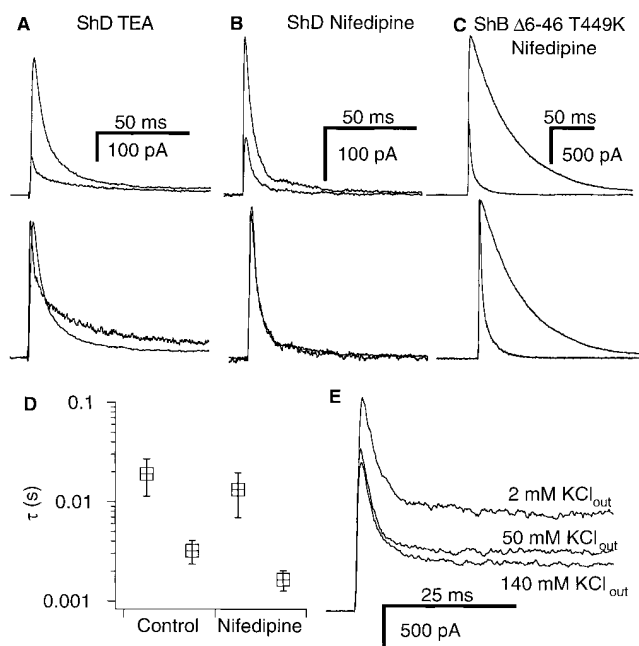


FIGURE 7. Nifedipine block in presence of either N- or C-type inactivation. (A) Internal TEA and N-type inactivation compete. The *ShD* currents obtained with and without internal TEA (2 mM) are shown at the top and the scaled currents are shown below. The scaled currents show a cross-over. (B) Nifedipine block of the macroscopic currents through *ShD* channels. (C) Macroscopic currents through the *ShBΔ6-46 T449K* channels with C-type inactivation (control) and 100 μ M nifedipine. Scaled currents (lower traces) show that the current in the presence of nifedipine inactivates faster. (D) Time constants of *ShBΔ6-46 T449K* current decline in control and in the presence of 100 μ M nifedipine in internal solution. The current traces were fitted with a sum of two exponentials. Each point represents mean \pm standard deviation of 12 experiments (Control) or 8 experiments (Nifedipine). (E) Nifedipine block (50 μ M) of the *ShBΔ6-46 T449V* currents without N- or C-type inactivation recorded with different external K⁺ concentrations. The currents were elicited by depolarizing pulses to +50 mV.

We further examined whether nifedipine and the C-type inactivation mechanism compete by using the *ShBΔ6-46 T449K* channel. This channel mutant has a 41-amino acid deletion in the amino terminus to disrupt N-type inactivation (Hoshi et al., 1990). In addition, it also contains a single amino acid mutation at position 449 from threonine to lysine to accelerate C-type inactivation (Lopez-Barneo et al., 1993). If C-type inactivation and nifedipine compete, it is expected that the time course of the current decline in the presence of nifedipine is slower because the nifedipine-bound channels are prevented from undergoing C-type inactivation. Representative currents through the *ShBΔ6-46 T449K* channels in control and in presence of 100 μ M nifedipine are shown in Fig. 7 C and rate constants obtained from two exponential current fits are shown in Fig. 7 D. In the presence of nifedipine (100 μ M), the current decline was faster than that in the absence of the drug. The results suggest that nifedipine and C-type inactivation do not compete.

If nifedipine induces the apparent inactivation in the *ShBΔ6-46 T449V* channel by enhancing the residual C-type inactivation mechanism, the nifedipine-induced inactivation should display properties similar to those of C-type inactivation. For example, the nifedipine-induced inactivation should slow down in the presence of high external K⁺ as C-type inactivation is slowed by high external K⁺ (Lopez-Barneo et al., 1993). We tested this hypothesis by comparing the efficacies of the nifedipine block in the presence of different external K⁺ concentrations. As compared in Fig. 7 E, the time course of the nifedipine block with 140 mM external K⁺ was not slower than that observed with 2 or 50 mM external K⁺. Thus, the nifedipine-induced inactivation and C-type inactivation differ in their sensitivities to external K⁺, suggesting that they are mediated by distinct mechanisms. This does not, however, rule out the possibility that nifedipine and C-type inactivation interact as suggested by the results obtained with the *ShBΔ6-46 T449K* channel (Fig. 7, C and D).

Voltage Dependence of the DHP Block

Because nifedipine is not a charged molecule, it was not expected that its action would be voltage dependent even if it might position itself in the membrane electric field. We examined the possible voltage dependence of the DHP action as shown in Fig. 8. The channels were opened first in response to a voltage pulse to +50 mV to allow the nifedipine molecules to block the channels. Then, the membrane voltage was changed to a variety of voltages where the steady-state probability of the channel being open is still saturated (Zagotta et al., 1994a). If the DHP action is voltage independent, the only instantaneous and time-independent changes in the current, reflecting the changes in the driving

force, should be observed, as recorded under control conditions (Fig. 8 A). The presence of the time-dependent relaxation of the current would suggest that the drug action is voltage dependent. The results in Fig. 8, B–D do show such time-dependent relaxation. The time courses of the current relaxations were fitted with single exponentials and the ratios of the extrapolated

instantaneous current amplitudes to the steady state currents are shown in Fig. 8 E. DHPs became less effective in blocking the current with greater depolarization. As a data description parameter for the voltage dependence, we chose the number of apparent equivalent charges (n). The value n was obtained by fitting the relative block data (Fig. 8 E) with the following function

$$\exp [n e (V - 50) / k T],$$

where V is membrane voltage, other constants have their usual meanings. Examples of this fit for 25 and 50 μM of nifedipine block of *ShB* Δ 6-46 T449V current are shown in Fig. 8 E as the solid lines. The apparent charge number is plotted against the DHP concentration in Fig. 8 F. The voltage dependence increased with the drug concentration. The data also show a difference in the voltage dependence of nifedipine and nimodipine blocks of the *ShB* Δ 6-46 T449V currents as well as a difference in the voltage dependence of nifedipine block of the *ShB* Δ 6-46 T449V current (Fig. 8 F, circles) and the *ShB* Δ 6-46 T449V:A463I current (squares).

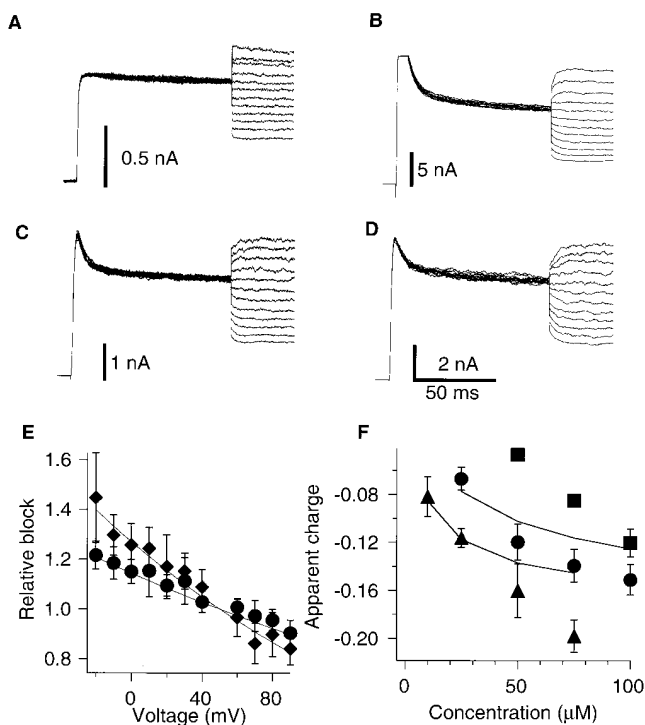


FIGURE 8. Voltage dependence of DHP block. The currents were first elicited by voltage pulses to +50 mV followed by voltage steps to -20 to +90 mV in 10 mV increments. (A) The *ShB* Δ 6-46 T449V channels do not show significant relaxation after the voltage change. (B) Slow relaxations of the macroscopic currents through the *ShB* Δ 6-46 T449V channels in presence of 25 μM nimodipine. The currents relaxed to lower values at voltages more negative than +50 mV showing more effective block of nimodipine at these voltages. (C) Same as B in presence of 100 μM nifedipine. (D) Voltage-dependent relaxation of the *ShB* Δ 6-46 T449V:A463I current with 100 μM nifedipine. (E) Voltage dependence of the block for *ShB* Δ 6-46 T449V in the presence of 25 μM (diamonds) and 50 μM (circles) of nifedipine. Relative block was calculated as a ratio of the current value immediately after the voltage change over the current value after relaxation based on the single exponential fit of the current decline. The data sets were fitted with function $\exp [n e (V - 50) / k T]$ to obtain number of equivalent charges n . (F) Concentration dependence of the equivalent charges associated with the voltage dependence of the block obtained as described in E for nifedipine block of the *ShB* Δ 6-46 T449V current (circles), *ShB* Δ 6-46 T449V:A463I current (squares), and nimodipine block of *ShB* Δ 6-46 T449V current (triangles). Solid lines represent the SCHEME v predictions of nifedipine (upper line) and nimodipine (lower line) block equivalent charges. The model predictions were calculated using the blocking rate constants obtained from the concentration dependence data shown in Fig. 4. Data points represent mean \pm standard deviation of five to eight experiments.

Nifedipine Interaction with Ion Flow through Channel

The results presented so far suggest that nifedipine interacts with the open state of the channel to impede the ion flow. One possible mechanism is that nifedipine physically occludes the ion conducting pore, acting as an open channel blocker. Several molecules have been shown to act as open channel blockers for voltage-dependent K^+ channels, including TEA, and the *Shaker* inactivation peptide (Demo and Yellen, 1991). If nifedipine acts as an open channel blocker, K^+ flux through the channel should regulate the block efficacy.

The effects of the direction of the net K^+ flow on the nifedipine efficacy are shown in Fig. 9. With 140 mM K^+ outside, the effects of nifedipine on the net inward and outward currents were measured at +30 mV with 0 mM and 140 mM K^+ inside, respectively. The reduction in the steady state current caused by nifedipine was not markedly altered by the changes in the net current flow, suggesting that DHP efficacy is independent of the direction of the net current flow.

Regulation of Nifedipine Efficacy by Mutation A463I in the S6 Segment

Residue 463 in the S6 segment of the *ShB* channel has been shown to be involved in regulation of C-type inactivation (Hoshi et al., 1991; Lopez-Barneo et al., 1993). In *ShB* Δ 6-46 T449V, where both the N- and C-type inactivation mechanisms are disrupted, mutating the residue 463 from A to I (*ShB* Δ 6-46 T449V:A463I) causes the mean open time and the single channel amplitude to increase (see Fig. 5). Consistent with the slow channel closing rate, the macroscopic G(V) curve of this chan-

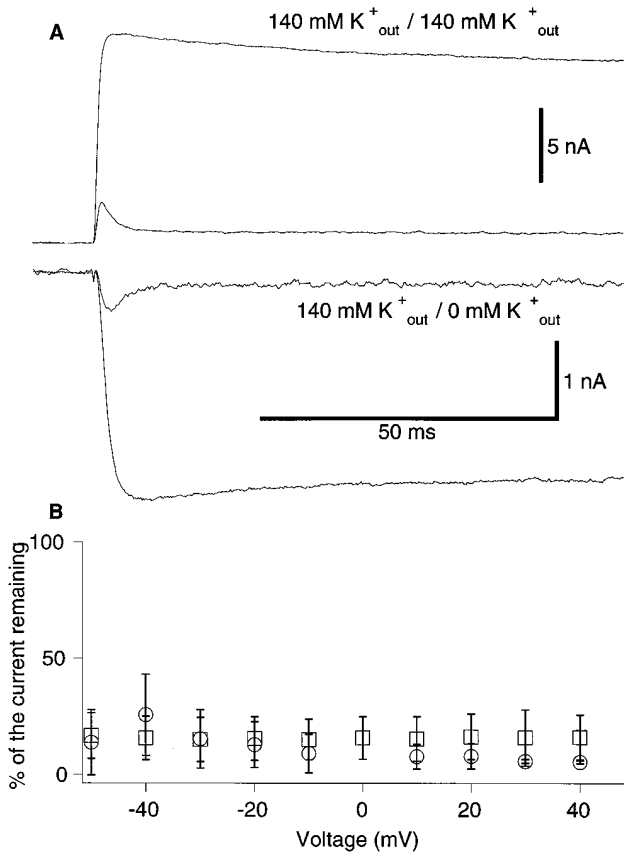
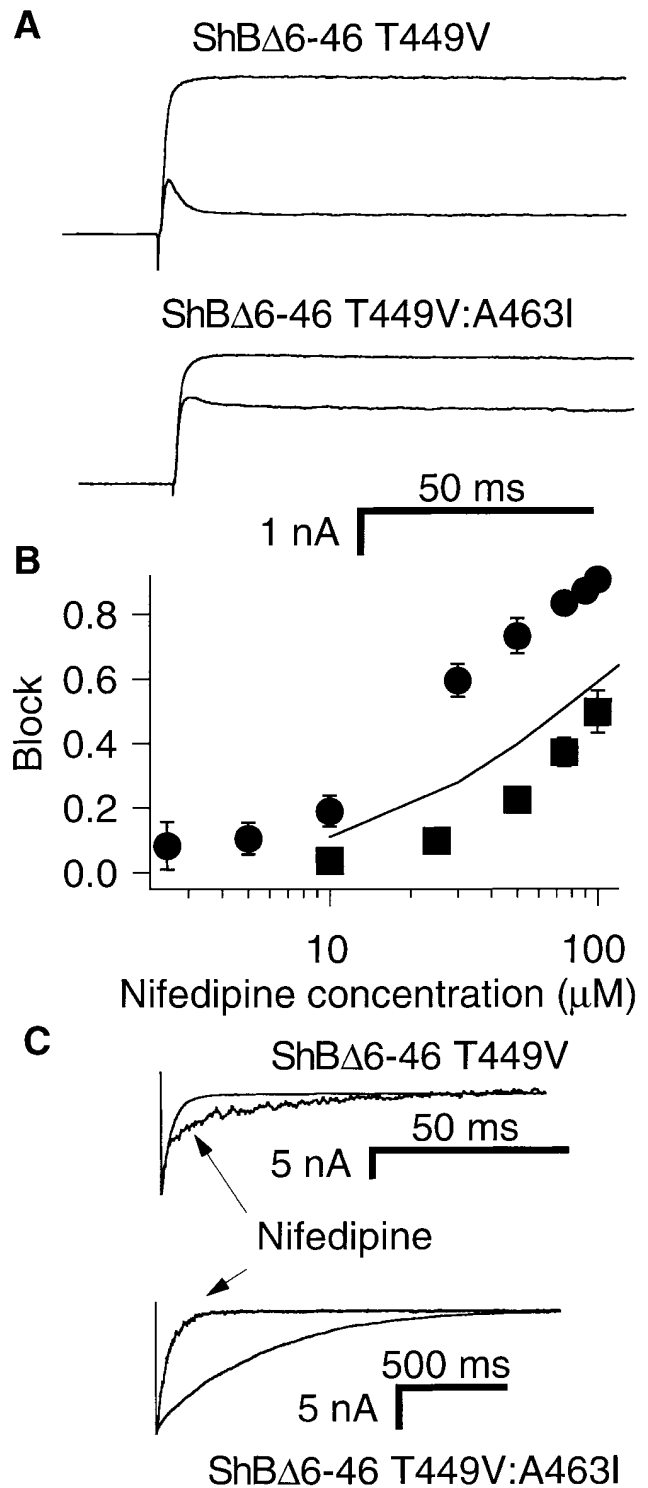


FIGURE 9. Nifedipine block does not depend on the current flow direction. (A) Macroscopic currents through the *ShBΔ6-46 T449V* channels (control and 100 μM nifedipine) at +30 mV. Outward currents were obtained with 140 mM $\text{K}^+_{\text{out}} / 140 \text{ mM } \text{K}^+_{\text{in}}$, and the inward currents with 140 mM $\text{K}^+_{\text{out}} / 0 \text{ mM } \text{K}^+_{\text{in}}$ (substituted with NMG). (B) Comparison of nifedipine block (100 μM) at various voltages with high K^+ (140 mM) inside (circles) and no K^+ ions inside (squares). External K^+ concentration was 140 mM in both cases. Data points show the fraction of unblocked current at the end of 200-ms voltage pulse to the voltages indicated (mean \pm standard deviation of five experiments).

nel is shifted to a more negative direction by ~ 40 mV compared with that of *ShBΔ6-46* or *ShBΔ6-46 T449V* (data not shown). We found that the effects of nifedipine on *ShBΔ6-46 T449V:A463I* are distinct from those on *ShBΔ6-46 T449V*. The effects of nifedipine on the *ShBΔ6-46 T449V:A463I* channel differ in three major

FIGURE 10. Differential effects of nifedipine on the *ShBΔ6-46 T449V* and *ShBΔ6-46 T449V:A463I* channels. (A) Effects of nifedipine (100 μM) on the macroscopic currents recorded at +50 mV from *ShBΔ6-46 T449V* (top) and *ShBΔ6-46 T449V:A463I* (bottom). (B) Comparison of the concentration dependence of nifedipine block of the *ShBΔ6-46 T449V* (circles) and *ShBΔ6-46 T449V:A463I* (squares) currents. Solid line represents the model prediction of SCHEME V for *ShBΔ6-46 T449V:A463I*. Values of the block



constants of nifedipine for *ShBΔ6-46 T449V* were used in simulation. *ShBΔ6-46 T449V:A463I* was simulated by stabilizing the open state in SCHEME I by 1.3 kcal/mol (see DISCUSSION). Each data point represents mean \pm standard deviation of six to nine experiments. (C) Scaled tail currents obtained at -100 mV for *ShBΔ6-46 T449V* and at -120 mV for *ShBΔ6-46 T449V:A463I* after pulses to +50 mV with 140 mM K^+_{out} . Nifedipine (100 μM) slowed the tail current in *ShBΔ6-46 T449V* and accelerated in *ShBΔ6-46 T449V:A463I*.

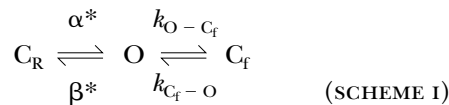
areas: steady-state fraction of the current blocked, recovery from the nifedipine block, and voltage dependence of the block. Nifedipine (100 μM) was less effective in blocking the *ShB* Δ 6-46 T449V:A463I channel than the *ShB* Δ 6-46 T449V channel. Fig. 10 *B* compares the concentration dependence of nifedipine block of the *ShB* Δ 6-46 T449V (*circles*) and *ShB* Δ 6-46 T449V:A463I (*squares*) currents, also showing that nifedipine is less effective on the *ShB* Δ 6-46 T449V:A463I channel. A difference in the voltage dependence of nifedipine block of these two channel types is shown in Fig. 8 *F* using the same symbols.

Nifedipine differentially altered the tail currents through the *ShB* Δ 6-46 T449V and *ShB* Δ 6-46 T449V:A463I channels. The tail current was slowed by nifedipine in the *ShB* Δ 6-46 T449V channels (Fig. 10 *C*). In contrast, nifedipine accelerated the time course of deactivation in the *ShB* Δ 6-46 T449V:A463I channel at all the voltages examined (-100 to -140 mV).

DISCUSSION

The results presented show that DHPs, such as nifedipine and nimodipine, reduce the ionic currents through the *Shaker* K^+ channels. Although higher concentrations of DHPs are required for inhibition of the *Shaker* channels than for some voltage-dependent Ca^{2+} channels (Hess, 1990), the DHP effects on the *Shaker* channels are still specific and require the intact dihydropyridine structure since the UV-treated DHPs largely lose their ability to reduce the current. The DHP efficacy is dependent on voltage, but independent of the direction of the K^+ flow.

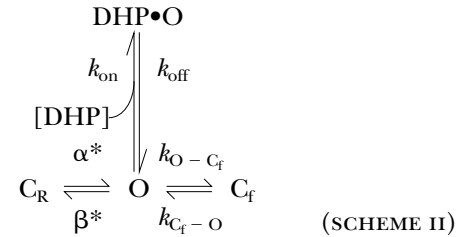
Our consideration of the possible biophysical and molecular mechanisms underlying the DHP action on the *Shaker* channels is based on the description of their gating process in Zagotta et al. (1994*b*). This scheme can be presented in a simplified form as the following:



where C_R represents the numerous closed states that the channel visits before opening, and C_f represents the closed state(s) that the channel enters after opening. At positive voltages, the value of the reverse rate constant β^* is small, and the fast transitions between C_f and O can be assumed to be at equilibrium. This scheme is well suited for the description of the gating behavior of *ShB* Δ 6-46 T449V, which shows neither C- or N-type inactivation in *Xenopus* oocytes (Hoshi et al., 1990; Lopez-Barneo et al., 1993). Although in this scheme, activation of the channel is represented with a single transition for simplicity, in numerical simulations we used the complete model as described in Zagotta et al.

(1994*b*). Values of all the rate constants were taken from Zagotta et al. (1994*b*). All the blocking schemes considered below are based on SCHEME I as they are reduced to this scheme in the absence of drug.

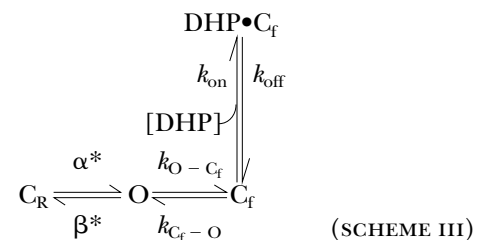
The simplest hypothesis that the DHP molecule binds to the open conducting state of the channel (O) only can be represented by the following scheme:



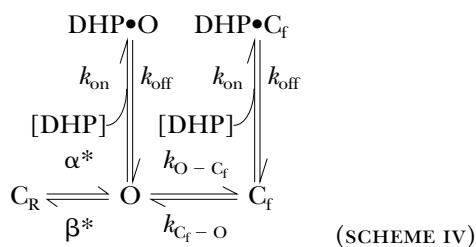
where $\text{DHP}\cdot\text{O}$ represents the drug-bound non-conducting open state. According to this scheme, the mean open time should decrease in a concentration-dependent manner in the presence of nifedipine. The mean open time was indeed decreased by nifedipine as shown in Fig. 5. Furthermore, according to SCHEME II, the reciprocal of the mean open time should be linearly related to the DHP concentration by the following relationship:

$$(1/t_{\text{open}}) = k_{\text{on}} [\text{DHP}] + k_{\text{O}-\text{C}_f}$$

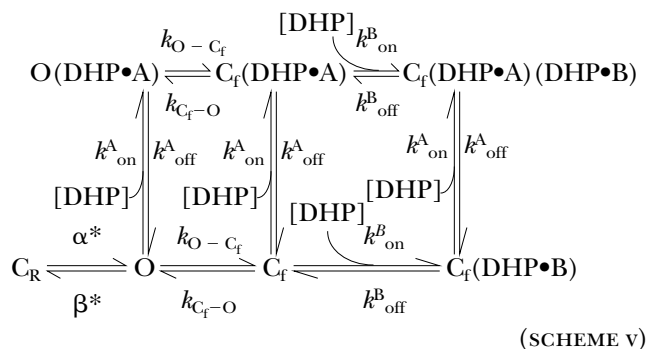
assuming that $\beta^* = 0$ at positive voltages. This linear prediction did not hold for all the concentrations of nifedipine examined (Fig. 5 *C*). At the high concentrations tested (>40 μM), the block was more than predicted, raising some implications about the nature of the block (see below). Furthermore, this model does not provide any mechanism for the voltage dependence of the block. To improve this model, we could assume that k_{on} and/or k_{off} in SCHEME II are voltage dependent. Alternatively, we can assume that the voltage dependence of the block comes from a voltage-dependent gating property of the channel rather than voltage-dependent binding of the drug. The rate constant for the C_f to O transition is voltage dependent (Zagotta et al., 1994*b*), decreasing with hyperpolarization with an equivalent charge of 0.17, similar to the voltage dependence of the DHP action (Fig. 8 *F*). Thus, the following scheme where the DHP molecule binds to the C_f state from which the exit rate constant γ is voltage dependent could be considered:



An obvious drawback of this scheme is that it does not predict the concentration-dependent mean open time. This can be resolved by combining SCHEMES II and III into a single scheme:



This model has four more free parameters than SCHEME I, and it can account for the effect of DHP on the mean open time and the voltage dependence of the block. This model, however, does not describe the concentration dependence curve well (Fig. 4) and it predicts a Hill coefficient of 1 as opposed to the observed higher value (Fig. 4 B), which requires binding of more than one DHP molecules to the channel. Thus, we introduce the following model:



Time constants of the current decline, voltage dependence of the block and the concentration dependence predicted by SCHEMES II and V were simulated and shown superimposed on the experimental data (Figs. 4, 8, and 10). For these computations, all parameters in the absence of drug were fixed at the values given in Zagotta et al. (1994b). Only the block/unblock rates included in each scheme were adjusted by minimizing the chi square value to simultaneously fit the steady-

state fractional block (Fig. 4, C and E) and the time constant of the current decline in the presence of nifedipine and nimodipine (Fig. 4, D and F). Table I shows the parameter values used to simulate the data. The predictions of SCHEME V with two DHP binding steps are markedly better than those of SCHEME II with one binding step (Fig. 4). With the rate constant values obtained from fitting the concentration dependence, SCHEME V also reproduces the voltage dependence of the block even though the parameters were not specifically optimized to fit the voltage dependence (Fig. 8 F).

The prediction of SCHEME V about the concentration dependence of the mean open time, which is determined by constant k_{on}^{A} , is consistent with initial slope of the curve shown in Fig. 5 C. The DHP binding to the fast closed state C_f in SCHEME V could also account for the non-linear concentration dependence of the mean open time (Fig. 5 C). Because the mean dwell time in C_f is short, many of the transitions to this state are unresolved. The DHP binding to the C_f state will increase its mean dwell time, thus reducing the number of unresolved events, which in turn could result in an apparent non-linear dependence of the mean open time on DHP concentration at high concentrations.

The model in SCHEME V can be used to explain the altered drug sensitivity of the *ShB* Δ 6-46 T449V:A463I channel. In the *ShB* Δ 6-46 T449V:A463I channel, the occupancy probability in the C_f state is lower than that in the *ShB* Δ 6-46 T449V channel as this channel has a greater mean open time. Thus, according to SCHEME V, where the second DHP molecule binds only to the C_f state, the *ShB* Δ 6-46T449V:A463I channel should be less sensitive to DHP than the *ShB* Δ 6-46 T449V channel, as opposed to SCHEME II, in which the increased occupancy of the open state should favor increased blocking. This prediction is consistent with the data obtained (see Fig. 10), where *ShB* Δ 6-46T449V:A463I is less sensitive to DHP. The shift in G(V) curve of the *ShB* Δ 6-46T449V:A463I channel could be well represented by decreasing the rate constants of the transitions away from the open state (δ and O- C_f as given in the model of Zagotta et al., 1994b) by a factor of 10, thus stabilizing the open state by ~ 1.3 kcal/mol. The concentration dependence given by SCHEME V with the block rates obtained from the *ShB* Δ 6-46T449V data with the open state stabilized by 1.3 kcal/mol is shown in Fig. 10 B. With those values, SCHEME V produces less block, which is consistent with the observed effect. We consider the results of the simulations based on SCHEME V to be in qualitative agreement with the observed effects. SCHEME V reasonably describes the concentration and voltage dependence of DHP block as well as the effect of A463I mutation. There are ways to further increase the agreement between the model prediction and the experimental data. For example, it is possible

TABLE I

Numerical Values of the Rate Constants Used to Simulate DHP Block

	SCHEME II		SCHEME V	
	nifedipine	nimodipine	nifedipine	nimodipine
k_{on} ($\text{s}^{-1} \mu\text{M}^{-1}$)	2.42	3.8	—	—
k_{off} (s^{-1})	31.9	24.1	—	—
k_{on}^{A} ($\text{s}^{-1} \mu\text{M}^{-1}$)	—	—	0.56	0.72
$k_{\text{off}}^{\text{A}}$ (s^{-1})	—	—	31.9	48.3
k_{on}^{B} ($\text{s}^{-1} \mu\text{M}^{-1}$)	—	—	49.4	72.2
$k_{\text{off}}^{\text{B}}$ (s^{-1})	—	—	229	109

to improve the general fit by assuming that allosteric interactions increase the rate of DHP binding to the C_f (DHP•B) state compared with that to the O state. Since the available experimental data did not specifically discriminate the various possible allosteric models, we did not consider them further.

The observation that DHP is not likely to compete with C-type inactivation (Fig. 6) suggests that C-type inactivation itself can occur from the fast closed state, which is stabilized by the bound DHP molecule. Because C-type inactivation is slowed by K⁺ ions (Lopez-Barneo et al., 1993; Baukrowitz and Yellen, 1996), it is reasonable to speculate that C-type inactivation occurs from the fast closed state or other nonconducting states (Baukrowitz and Yellen, 1996). Baukrowitz and Yellen (1996) showed that internal TEA derivatives enhance C-type inactivation by emptying K⁺ ions from the pore. Similarly, DHP may have two effects on the *Shaker* channel. First, binding of the DHP itself to the channel decreases the K⁺ flux. The reduced K⁺ ion occupancy in the channel pore in turn increases the residual C-type inactivation rate.

Although a mutation in the S6 segment affected the DHP efficacy (Fig. 10), it is likely that other amino acid residues are also involved in regulating the DHP efficacy. For example, considering that DHP preferentially works from the intracellular side, the residues in the internal mouth of the channel may also be involved. Those amino acid residues in the S5, P, and S6 segments shown to be important for the 4-aminopyridine block (Kirsch et al., 1993) are also good candidates for involvement in the DHP sensitivity. However, since the DHP efficacy does not appear to be markedly influ-

enced by the net K⁺ flux (Fig. 9), the DHP molecules probably do not lie directly in the K⁺ flux pathway.

A large number of dihydropyridines have been synthesized and these drugs appear to have different potencies in blocking the *Shaker* potassium channel (Fig. 3; unpublished observation). At the same concentration, nimodipine appears to be more effective than nifedipine (Fig. 4). However, minor quantitative changes in the rate constants of the model in SCHEME V can account for the observed differential potency, suggesting that the underlying biophysical mechanisms are the same. Furthermore, by comparing the effects of different dihydropyridines with different side groups, it should be possible to gain information on which structural components of the drug are critical in determining the different rate constants of the model.

Several μM of nifedipine was required to observe noticeable effects on the *ShB* channel currents at +50 mV whereas the drug's effects on L-type Ca²⁺ channels may be observed in the nM range. Despite the higher concentration required to affect the *Shaker* potassium channels, the effects are specific in that the UV-treated nifedipine is ineffective. Because the DHP action on *Shaker* channels is enhanced by hyperpolarization, it is possible that the efficacy may be greater under physiological conditions where the transmembrane potential may not reach +50 mV. It is also possible that other homologues of *Shaker* channels show much higher DHP sensitivities than the channels examined in this study. Given the structural similarities found in different voltage-dependent ion channels, it will be interesting to rigorously compare the biophysical and molecular mechanisms of the DHP action on voltage-dependent Ca²⁺ and K⁺ channels.

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