Voltage-Dependent Block of Calcium Channel Current in the Calf Cardiac Purkinje Fiber by Dihydropyridine Calcium Channel Antagonists

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SUMMARY. We have investigated the mechanisms of blockade of calcium channel current by the dihydropyridines, e.g., nisoldipine, nitrendipine, and nicardipine. Membrane current was recorded in isolated calf Purkinje fibers using a two-microelectrode voltage-clamp technique, and voltage protocols were designed to identify voltage- and use-dependent block by these compounds systematically. Our results show that calcium channel blockade by dihydropyridine derivatives is strongly modulated by membrane potential. Block is more pronounced when current is measured from depolarized holding potentials, but in contrast to verapamil, this voltage-dependent block occurs in the absence of repetitive depolarizations. Use-dependent block by dihydropyridines is observed at pulse frequencies greater than 1 Hz. Our results suggest that dihydropyridines bind preferentially to the inactivated state of the calcium channel, and that the development of use-dependent block is related to the ionization constants of the compounds. Furthermore, binding is approximately one thousand times stronger to inactivated channels than to resting channels. This state-dependent difference in binding affinities may account for the previously reported contrast between electrophysiological and binding data for these compounds. (Circ Res 55: 336-348, 1984)

A major new class of drugs collectively referred to as the organic Ca channel antagonists (or blockers) has been investigated intensely by both electrophysiological and biochemical techniques. These compounds have proven clinically useful in the treatment of angina, hypertension, and certain cardiac arrhythmias (Singh et al., 1983; Schwartz and Taira, 1983).

On the basis of electrophysiological and binding studies, at least three distinct classes of Ca channel antagonists have been identified (for review, see Schwartz and Triggle, 1983; Nayler and Horowitz, 1983). An important pharmacological finding is that the prototypic Ca channel antagonists, the papaverine derivatives (e.g., verapamil and D600) block Ca channels of the myocardium in a voltage- and use-dependent fashion (Ehara and Kaufmann, 1978; Kohlhardt and Mnich, 1978; Pelzer et al., 1982). Diltiazem, a benzothiazepine derivative also inhibits Ca current in a voltage- and use-dependent manner (Tung and Morad, 1983; Lee and Tsien, 1983), and, similar to verapamil, is thought to block Ca channels preferentially in the inactivated state (Kanaya et al., 1983). In contrast, voltage-clamp studies on the third class of Ca channel antagonists, the dihydropyridine derivatives (e.g., nifedipine, nitrendipine, nisoldipine), have suggested that these compounds exhibit little, if any, voltage- or use-dependent block of Ca current in the heart (Kohlhardt and Fleckenstein, 1977; Bayer and Ehara, 1978; Kass, 1982; Tung and Morad, 1983).

A model has been proposed (modulated receptor hypothesis) that can account for the voltage- and use-dependent block of Na channels by local anesthetics (Hille, 1977; Hondeghem and Katzung, 1977). According to this model, charged tertiary amine local anesthetics can reach their receptors inside the channel by a hydrophilic pathway available only when the channel gates are open, and hence are characterized by significant use-dependent block. In contrast, permanently uncharged anesthetics (e.g., benzocaine) can reach the same receptor via a hydrophobic region of the membrane and, therefore, only show use-dependent effects on Na current during rapid stimulation, but do shift the steady state inactivation curve to more negative potentials (Hille, 1977). The channel state-dependent block of Ca channels by verapamil, D600 (pKₐ = 8.7) and diltiazem (pKₐ = 8.1) (Rodentkirchen et al., 1982) resembles the block of Na channels by tertiary amine local anesthetics in nerve (Hille, 1977; Schwarz and Vogel, 1977) and heart (Hondeghem and Katzung, 1977; Bean et al., 1983), and is consistent with the modulated receptor scheme.

Ca channel blockade by the dihydropyridines has been described as being similar to the effect of tetrodotoxin on the Na channel (Nayler and Horowitz, 1983), since one of these compounds, nifedipine, was reported to have no frequency-dependent ef-
fects and did not alter the kinetics of inactivation or recovery from inactivation of Ca current in voltage-clamped ventricular myocardium (Kohlhardt and Fleckenstein, 1977). However, nifedipine does inhibit the maximum upstroke velocity of action potentials recorded from AV-nodal cells in a frequency-dependent fashion (Kohlhardt and Haap, 1981).

We have reinvestigated the mechanisms of action of dihydropyridine derivatives using voltage clamp protocols designed to identify voltage- and use-dependent block of Ca-channel current by them more systematically. We studied the dihydropyridines, nisoldipine, nitrendipine (pK$_a$ < 3.5) (Rodentkirchen et al., 1982) and nicardipine (pK$_a$ = 7) (L. Sanders, personal communication). The new finding presented in this paper is that dihydropyridines block Ca channel current in a voltage-dependent manner. The results suggest the compounds have a high affinity for the inactivated state of the channel, and that the relative contribution of use-dependent block can be predicted based on the calculated percent ionization of these compounds at physiological pH.

Some of these results have been reported briefly to the Biophysical Society (Sanguinetti and Kass, 1984).

**Methods**

Calf hearts were obtained from a local slaughterhouse and transported to the laboratory in cold (4°C), oxygenated Tyrode's solution. Free-running Purkinje fiber bundles (100–200 μm in diameter) were dissected from either ventricle and shortened to 0.5–1.5 mm.

Membrane current was measured by a conventional two-microelectrode technique described elsewhere, with electrode spacing appropriate for measurement of inward current (Kass et al., 1979; Kass and Bennett, in press). Membrane current and voltage records were recorded, stored, and analyzed on a PDP 1123 computer (Digital Equipment Corp.). The voltage-recording electrodes (10 MΩ) were filled with 3 M KCl. Current-passing electrodes (30–40 MΩ) were filled with 1.5 M tetrabutylammonium chloride (TBA) (Sigma). Electrodes were bevelled as described in another paper (Kass and Bennett, in press). Iontophoresis of TBA was performed during all the experiments to block the two time-dependent outward currents (transient outward, $I_{o}$ and delayed rectifier, $I_{r}$) that interfere with measurement of Ca channel current in these preparations (Kass et al., 1982). In most experiments, Ca was replaced by Sr in the superfusate to increase the magnitude and stability of current through the Ca channel. In addition, substitution of Sr for Ca eliminates Ca-dependent inactivation of the channel that complicates the analysis of voltage-dependent mechanisms (Lee and Tsien, 1983; Kass and Sanguinetti, in press). Because Ca is not always the permeant divalent cation in solutions used in this study, we refer to current through this pathway as Ca channel current. The voltage-dependent effects of the drugs on Ca channel current was the same, regardless of whether Sr or Ca was the permeant divalent cation. The replacement of Ca by Sr was done only after substantial decreases in outward currents by iontophoresis of TBA.

Na current was eliminated by holding the membrane potential positive to −50 mV to inactivate the Na channels (Lee et al., 1979; Colatsky, 1980), or by addition of 10 μM tetrodotoxin (TTX) (Calbiochem).

Experiments in the presence of blockers were carried out after steady state effects on Ca channel current had been obtained for a given drug concentration. These effects were monitored continuously during exposure to the drugs by measuring Ca channel current in response to depolarizing voltage pulses applied at a low frequency (≤ 0.2 Hz). Exceptions to this protocol are specifically noted in the text. Steady state effects of the drugs were attained within 15–20 minutes of a solution change. The mixing time for our experimental chamber was 2–3 minutes.

In some experiments, the fraction of Ca channel current blocked at a given voltage by a given drug concentration was determined as described in an earlier study (Kass, 1982). The procedure required measurement of current in the absence of drug, in the presence of an intermediate drug concentration (~200 nM), and then in the presence of a very high drug concentration (10 μM) that completely blocks all Ca channel current and reveals background (leak) current. The background current is then subtracted from the control (drug-free) and intermediate concentration drug records to determine Ca channel current available in control and remaining in the presence of the intermediate drug concentration.

The standard Tyrode's solution had the following composition (mM): NaCl (150), KCl (5), CaCl$_2$ (5.4), MgCl$_2$ (0.5), glucose (5), Tris (10); pH 7.4. All solutions were gassed with 100% O$_2$. Temperature was maintained at 37°C (±0.5°C) with a thermoelectric device (Cambion). Nisoldipine and nitrendipine, kindly supplied by Miles Laboratories, were dissolved in polyethylene glycol (PEG) 400 to make a 10 mM stock solution. PEG 400 has no effect on Ca channel current at a concentration 50 times higher than that used in the present study (Kass, 1982). Nicardipine and verapamil were dissolved in distilled water. Aliquots of these stock solutions were added to the Tyrode's solution to obtain the final desired concentrations. Nicardipine was a generous gift from Syntex, and verapamil was kindly supplied by Knoll Pharmaceutical.

**Curve-Fitting Procedures**

Theoretical curves were fit to the experimental data using the algorithm of Marquardt (1963) as described by Bevington (1969). This procedure allowed us to fit the data with an exponential function with one or two time constants plus a baseline, as well as with an expression for steady state inactivation: $(1 + \exp(V - V_h)/k)^{-1}$.

Data are expressed as means ±SEM ($n$ = number of experiments). Tests of significance were performed using paired Student's t-test where appropriate.

**Results**

Nisoldipine Block of Ca Channel Current: Influence of Depolarization Frequency

Previous studies have suggested that block of Ca channel current by dihydropyridine derivatives is not modulated by the frequency of applied depolarizing voltage steps (Kohlhardt and Fleckenstein, 1977). To test this suggestion more completely, we have studied the effects of pulse frequency on nisoldipine block over a more extensive frequency
range. When membrane potential is held at sufficiently negative voltages (near −70 mV) and depolarizing pulses are applied at low frequencies (< 0.5 Hz), we confirm the previous observations: there is little or no block of current, regardless of how many pulses are applied. However, when pulse frequency is increased, marked block develops in the steady state (Fig. 1), and this block is relieved when pulse frequency is lowered.

Several mechanisms exist that can possibly account for such frequency-dependent changes in block, and in the remainder of this paper we test for these mechanisms. Our first test was to determine whether this effect is caused by a nisoldipine-induced prolongation of the time course for recovery from apparent inactivation of Ca channel current.

Effects of Nisoldipine on Recovery from Inactivation

The time course of the recovery from inactivation was investigated using a standard double-pulse protocol (insets, Fig. 2). Control experiments in a previous study had established that in the absence of drug, the time course of recovery is best fitted by a function with one exponential component when the conditioning prepulse is brief (50 msec) and by a function with two exponential components when the prepulse is longer (500 msec) (Kass and Sanguinetti, unpublished data).

![Figure 1](image1.png)

**Figure 1.** Influence of pulse frequency on Ca channel current blockade by nisoldipine. Inset: membrane current in response to 50-msec voltage steps to 0 mV at pulse frequencies of 0.1 Hz and 5.0 Hz in the absence (○) and presence (●) of nisoldipine (200 nm). Plot shows steady state measures of peak inward current during voltage pulses to 0 mV against pulse frequency in the absence (○) and presence (●) of nisoldipine. 5.4 mM Sr**⁺** Tyrode’s solution + 10 μM TTX. Holding potential = −70 mV. Preparation 318–1.

![Figure 2](image2.png)

**Figure 2.** Influence of nisoldipine on the time course of recovery from inactivation. Insets show voltage protocols. Part a: recovery of current after 50-msec prepulse to 0 mV. Peak inward current measured during test pulse to 0 mV at variable time (t) after prepulse plotted against t in the absence (○) and presence (●) of nisoldipine (200 nm). No further recovery of current occurred for interpulse intervals of 2–4 seconds in the presence of nisoldipine in this and the other three experiments. Time constant for recovery: τ1 = 23 msec (control), 18 msec (drug). Part b: recovery of current after 500-msec prepulse to 0 mV. Time constants for recovery: τ1 = 330 msec, τ2 = 760 msec (drug). Smooth curve and time constants determined as in methods. 5.4 mM Sr**⁺** Tyrode’s solution + 10 μM TTX. HP = −70 mV. Preparation 317–1.

We find that nisoldipine has very little influence on the time course of recovery from inactivation after brief (50-msec) pulses (Fig. 2a). In four experiments, the time constant for recovery after 50-msec pulses (τ1) was not measurably affected by the drug (200 nm) (control τ1 = 20 ± 5 msec; nisoldipine τ1 = 16 ± 3 msec). A second slow component of recovery was not observed before or after addition of drug when the prepulse was 50 msec. In contrast, nisoldipine slowed the recovery from inactivation after 500-msec pulses in each of three experiments in which this effect was tested (Fig. 2b). Thus, although nisoldipine does measurably slow the repriming kinetics of Ca channel current after longer prepulses, the results of the experiments with the brief prepulses suggest that, after a single 50-msec
depolarizing pulse, only a very small fraction of current recovers slowly at the holding potential. This fraction of current is too small to be resolved in our experiments. During repetitive pulsing, however (such as shown in Fig. 1), this small effect becomes additive and produces more block of Ca channel current in the steady state at briefer interpulse intervals.

Nisoldipine Block of Ca Channel Current: Influence of Holding Potential

The next possibility we tested is that changes in membrane potential, even in the absence of repetitive activity, can alter the effectiveness of current blockade by nisoldipine. In this test, membrane current was recorded during a single depolarizing voltage step applied from a $-70 \text{ mV}$ or a $-45 \text{ mV}$ holding potential after a 2-minute pulse-free interval at either holding potential. The results of one of these experiments are shown in Figure 3.

In the absence of drug, a change in the holding potential from $-70$ to $-45 \text{ mV}$ results in some (25%) decrease in inward current because of slow inactivation of the Ca channel (Kass and Scheuer, 1982). Following the control measurements, the superfusate was changed to a nisoldipine (200 nM)-containing solution, and after steady state was reached in this solution (see Methods), membrane current was again recorded during single test pulses from the two holding potentials. The current recorded during the pulse applied from the $-70 \text{ mV}$ holding potential in the presence of the drug was only slightly less inward than that measured for control (Fig. 3a), indicating that very little block had occurred. However, when current was measured from the depolarized holding potential ($-45 \text{ mV}$), almost all inward current was blocked (Fig. 3b). Subsequent return to the $-70 \text{ mV}$ holding potential for 2 minutes before repeating a current measurement in this and other experiments resulted in recovery of inward current (measured as maximum inward current during the test pulse) to $81 \pm 6\%$ ($n = 7$) of its control level. These results clearly show that block of Ca channel current by nisoldipine can be modulated by changes in membrane potential in the absence of repetitive activity.

We next determined the time course of development of or recovery from block that occurs after holding potential is changed. The approach that we chose is shown in Figure 4. Membrane current was measured during a single test pulse applied at variable times after changing the holding potential. Membrane potential was then returned to the initial holding potential for 2 minutes before repeating a measurement.

In the experiment illustrated in Figure 4, control measurements showed no change in current when these procedures were followed in the absence of drug. Thus, in contrast to the results shown in Figure 3, in this experiment there was very little slow inactivation in the absence of drug. However, when the holding potential was changed from $-70$ to $-45 \text{ mV}$ in the presence of nisoldipine (200 nM), inward current was almost completely blocked after 20 sec-

![Figure 3](https://example.com/figure3.png)

**FIGURE 3.** Holding potential influences Ca channel current blockade by nisoldipine. Membrane current in response to voltage pulse to 0 mV applied from $-70 \text{ mV}$ holding potential (part a) and $-45 \text{ mV}$ holding potential (part b) in the absence (C) and presence (N) of nisoldipine (200 nM). Pulses were applied after 2-minute pulse-free interval at each holding potential. 5.4 mM Sr$^{**}$ Tyrode’s solution + 10 μM TTX. Preparation 317–1.

![Figure 4](https://example.com/figure4.png)

**FIGURE 4.** Time course of the onset of and recovery from block by nisoldipine. Maximum inward current was measured during a test pulse to 0 mV at variable times (t) and plotted against t after the holding potential was changed from $-70$ to $-45 \text{ mV}$ (onset, $\bullet$) and from $-45$ to $-70 \text{ mV}$ (recovery, $\triangle$). Inset: voltage protocols and current traces in response to test pulses 20 msec and 60 seconds after the holding potential was changed. See text for details. Curves: exponential functions fitted to the data. Onset: $r_1 = 1.3 \text{ sec}$, $r_2 = 5.1 \text{ sec}$; recovery: $r_1 = 1.5 \text{ sec}$, $r_2 = 30 \text{ sec}$. Control measurements (not shown) indicated no time-dependent changes in maximum inward current under these conditions. 5.4 mM Sr$^{**}$ Tyrode’s solution + 10 μM TTX. Preparation 323–3.
onded at the depolarized voltage. The onset of block followed a biexponential time course in this and four other experiments ($\tau_1 = 2.5 \pm 0.6$ sec; $\tau_2 = 15.3 \pm 2.2$ sec, $V_{H} = -45$ mV). Current that had been blocked when the holding potential was depolarized could be recovered when the holding potential was returned to $-70$ mV (▲). Recovery also was biexponential in this and other experiments.

Voltage-Dependent Modulation of Nisoldipine Block: Relative Contribution of Repetitive Activity and Holding Potential

The results presented so far show that Ca channel current blockade by nisoldipine can be markedly altered by changes in holding potential at voltages between $-70$ and $-45$ mV. At these voltages, drug-free cardiac Ca channels are predominantly in the rested state (Reuter, 1979; Reuter et al., 1982; Kass and Sanguinetti, in press). Since Lee and Tsien (1983) have reported open channel block of Ca channels by dihydropyridines in isolated ventricular cells, we designed experiments to determine: (1) whether this effect can be seen in our preparations in addition to what we will refer to as “holding-potential”-dependent block and (2) if observed, its relative contribution to the blocking of Ca channels by nisoldipine at different membrane potentials.

Drugs that bind to the activated (open) state of ion channels have been shown to increase the relaxation (apparent inactivation) rate of current through these channels during initial depolarizing voltage pulses after exposure to the drug (Yeh, 1982; Lee and Tsien, 1983). We performed this experiment in our preparations as a test for evidence of open channel block by nisoldipine. The procedure for this experiment differs from that for all other experiments in this study, in that no depolarizing pulses were applied during the exposure to nisoldipine. Instead, a control record of current in response to a single voltage pulse was compared with current recorded in response to an identical pulse applied 15 minutes after changing to a nisoldipine (200 nM)-containing solution. The holding potential was maintained at $-45$ mV throughout the experiment. As shown in Figure 5, inward current measured in the presence of the drug is reduced during this single pulse, and the rate of apparent inactivation is increased, consistent with the observations of Lee and Tsien (1983). We observed an increase in the rate of apparent inactivation of Ca channel current during single voltage pulses in each of four preparations in which this protocol was followed (Table 1).

We next designed experiments to determine the relative contributions of pulse-dependent and holding potential-dependent modulation of block by nisoldipine. The protocol used and the results of one experiment are shown in Figure 6. Blockade of current is expressed as the fraction of inward current blocked relative to that measured during control (see Methods).

The onset of block in the experiments shown in Figure 6 was first measured using a single pulse protocol, after changing from a $-70$ to a $-45$ mV holding potential (●). These results were compared with the development of block that occurred when a train of brief pulses (20 msec) was imposed along with the change in holding potential (▲). The block that developed was nearly identical for each protocol. Thus, the absence or presence of brief depolarizing pulses applied from a depolarized holding potential does not markedly influence current blockade by nisoldipine. However, when the duration of each pulse applied during the train is increased to 200 msec, the amount of block is enhanced (●). Comparison of the fraction of current blocked by nisoldipine in each of these protocols was carried out in six preparations. The results of these experiments are summarized in Table 2 which shows the fraction of current blocked by the drug after 30 seconds at the depolarized holding potential during each protocol.

In these experiments, the primary factor in modulation of nisoldipine’s blocking action is clearly the holding potential. Nevertheless, additional block is observed during repetitive pulsing when pulse duration is increased (Table 2). If this increased blockade is related to the opening of channels during the repetitive activity, then the amount of block should be affected by the fraction of channels that open during each depolarizing voltage step. To test for...
TABLE 1

<table>
<thead>
<tr>
<th>Preparation</th>
<th>% Decrease in peak current</th>
<th>Holding potential (mV)</th>
<th>Test potential (mV)</th>
<th>% Decrease in time constants, ( \tau )</th>
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</thead>
<tbody>
<tr>
<td>322-1</td>
<td>71%</td>
<td>-40</td>
<td>+14</td>
<td>( \tau_1 ) 42% ( \tau_2 ) 36%</td>
</tr>
<tr>
<td>318-1</td>
<td>48%</td>
<td>-45</td>
<td>+14</td>
<td>( \tau_1 ) 26% ( \tau_2 ) 17%</td>
</tr>
<tr>
<td>319-1</td>
<td>26%</td>
<td>-45</td>
<td>+10</td>
<td>( \tau_1 ) 20% ( \tau_2 ) 6%</td>
</tr>
<tr>
<td>317-1</td>
<td>15%</td>
<td>-50</td>
<td>-1</td>
<td>( \tau_1 ) 27% ( \tau_2 ) 32%</td>
</tr>
</tbody>
</table>

Average \( 29 \pm 4.7\% \) \( 23 \pm 6.9\% \)

* \( P < 0.005 \)
† \( P < 0.05 \)

this possibility, a train protocol was designed in which the voltages of the repetitive test pulses was varied from -24 to +24 mV. These voltages were chosen because, in the steady state, the probability of drug-free channels being open is near 0 at -24 mV and 1 at +24 mV (Reuter, 1979; Kass and Sanguinetti, in press). As shown in Figure 7, there is additional block that develops when the repetitive pulses are applied to more positive voltages, but this effect is very small when compared to the overall blocking activity of the drug. Similar experiments were repeated in two other preparations, and these results were confirmed in each case.

Comparison to Other Blockers: Effects of Ionization of Drug

Verapamil

Other organic compounds such as D600, verapamil, and AQA 39 are known to block Ca channel current in a voltage-dependent manner (Pelzer et al., 1982; Trautwein et al., 1983). However, for these compounds, changes in holding potential alone do not result in block of current during a single voltage pulse. Block increases only after several depolarizing pulses have been applied from the more positive holding potential, regardless of the time spent at this voltage before a test pulse is applied (see Pelzer et al., 1982, Fig. 3). Because this is in such sharp difference, the relative importance of holding potential- and pulse-dependent block was studied with verapamil. As shown in Table 2, the fraction of block that develops after applying a single voltage pulse is small compared to the fraction that develops after applying repetitive voltage pulses. The voltage clamp protocols are shown in Figure 7 and described in the text. Train protocols: data indicate fraction of peak inward current blocked by the 15th pulse of a train of 20-msec or 200-msec voltage pulses to 0 mV (interpulse interval, 2 seconds) after changing to the indicated holding potential (HP). Single pulse protocol: fraction of block that developed after change to indicated HP assayed by single voltage pulse applied 30 seconds after change in HP. Total number of preparations (six) include four experiments in Sr ++ solutions and two experiments in Ca ++ solutions.

* Not significantly different from single pulse block.
† Significantly different from 20-msec pulse train block (\( P < 0.05 \)), and from single pulse block (\( P < 0.001 \)).
FIGURE 7. Development of block by nisoldipine during repetitive pulsing: influence of pulse voltage. Voltage protocol (inset): train of 100-msec voltage pulses was applied 10 msec after change in holding potential from -72 to -45 mV. First (P1) and last (P10) pulses were applied to 0 mV; pulses P2 through P9 were applied to variable voltages (Vt). Maximum inward current was measured during P1 and P10 in the absence (open bar) and presence of nisoldipine (shaded bar) for three different test conditions: Vt = -24 mV, +12 mV, and +24 mV. Interepulse interval was 2 seconds, in each case. 5.4 mM Sr** Tyrode's solution + 10 µM TTX. Preparation 343-1.

FIGURE 8. Verapamil: relative importance of holding potential- and pulse-dependent block. Same protocols as described in Figure 6, but plot shows maximum inward current during measured test pulses for each protocol rather than fractional block. Inset shows current traces in response to first (P1) and last (P10) voltage pulse during train protocol in the absence (open symbols) and presence (filled symbols) of verapamil (500 nM) for trains of 200-msec pulses (C,C) and 20 msec pulses (A,A). Maximum inward current measured during test pulse applied 30 seconds after holding potential was changed from -70 mV to -45 mV is also plotted in the absence (O) and presence of drug (M). 5.4 mM Sr** Tyrode's solution + 10 µM TTX. Preparation 330-1.

contrast to our observations with nisoldipine, we carried out experiments comparing the relative influence of holding potential and repetitive activity on current blockade by verapamil.

The contrast between block by nisoldipine and verapamil is very evident in the results shown in Figure 8. Using the single pulse protocol, we find that in the presence of verapamil there is no change in inward current measured during a test pulse even 30 seconds after the holding potential has been changed from -70 to -45 mV (■). However, block does develop when the change in holding potential is accompanied by a train of brief (20 msec) depolarizing pulses (▲), and block is enhanced when the duration of these pulses is prolonged (●). These results suggest that Ca channels must first open before they can be blocked by verapamil, because Ca channels open rapidly during voltage steps to 0 mV, and the probability of these channels opening is much greater at 0 mV than at -45 mV. Similar results obtained in ferret papillary muscle have been cited as evidence that verapamil preferentially sta-

bilizes drug-bound channels in the inactivated state of the Ca channel (Kanaya et al., 1983).

Verapamil (pKs = 8.7) exists almost entirely in the charged form at pH 7.4, and nisoldipine (pKs < 3.5) exists almost entirely in the neutral form at this pH. Thus, it is possible that the contrast in holding potential modulation of block by these two compounds might be due to differences in their relative hydrophilicity, and not to other structural dissimilarities. That is, because verapamil is in the charged form at physiological pH, perhaps it can only interact with its receptor via the hydrophilic pathway of an open Ca channel. To test for this possibility, we subjected two other compounds to similar procedures.

Nitrendipine

Nitrendipine is a dihydropyridine and, like nisoldipine, exists almost entirely in the neutral form at physiological pH. We find that block by this drug responds to changes in membrane potential in a
manner that closely resembles nisoldipine. Block is largely due to changes in holding potential (Table 3).

Nicardipine

Nicardipine \( (\text{pK}_a = 7.0) \) is a dihydropyridine that is 28% ionized at pH 7.4. If the major difference between the blocking activity of verapamil and nisoldipine (and nitrendipine) is related to the relative hydrophilicity of the compounds, then block by nicardipine ought to be intermediate to these other drugs in its response to changes in membrane potential. As shown in Figure 9, nicardipine resembles nisoldipine (and nitrendipine) in that block increases after changing holding potential to a depolarized level (\( \Delta \)). But unlike these other dihydropyridines, block by nicardipine increases dramatically when brief (20-msec) repetitive depolarizing pulses are applied from the new holding potential (\( \Delta \)). As was the case for the other compounds, increasing pulse duration during the train of pulses increases the amount of current blocked (\( \bullet \)). These results provide evidence that the differences between block by verapamil and the dihydropyridines may be related to the degree of ionization of these compounds.

Voltage-Dependence of Nisoldipine Block:
Effects on Steady State Availability of Ca Channel Current

The results presented so far have shown that changes in holding potential dominate the modulation of Ca channel block by nisoldipine. In this section, we examine the manner in which this modulation varies with membrane potential. Availability of Ca channel current was characterized by measuring current during voltage pulses to a common test potential after applying conditioning pulses to different potentials. Ca channels inactivate during the conditioning prepulse in the absence of drugs. In the presence of drugs, block of current that develops during a given prepulse will be detected as extra apparent inactivation that had occurred during that prepulse. Thus, these experiments essentially test for drug-induced changes in the availability of Ca channel current as a function of voltage.

When the duration of the conditioning pulses is 500 msec, we find that the inactivation curve is not changed by the drug \( (n = 2) \), indicating that little additional block by the drug develops or is removed in this time.

In contrast, when the conditioning pulses are increased to 30 seconds, the drug has pronounced effects (Fig. 10). The control, drug-free inactivation curve differs slightly from the 500-msec curve, as expected, because of the presence of slow inactivation \( (\text{Kass and Scheuer, 1982; Scheuer, 1983}) \). However, in the presence of nisoldipine \( (200 \text{ nM}) \), apparent inactivation of current occurs at much more negative potentials; the drug caused a 21-mV hyperpolarizing shift in the steady state inactivation curve. Doubling the nisoldipine concentration resulted in only an additional 2-mV shift of this relation. The results for this and other steady state inactivation experiments are summarized in Table 4.

### Discussion

The results of this study show that blockade of Ca channel current by dihydropyridine derivatives

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### Table 3

<table>
<thead>
<tr>
<th>Holding potential</th>
<th>Nitrendipine</th>
<th>Nicardipine</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mV)</td>
<td>Single pulse</td>
<td>20-msec pulse train</td>
</tr>
<tr>
<td>(-45 (n = 4))</td>
<td>0.53 ± 0.03</td>
<td>0.51 ± 0.06*</td>
</tr>
<tr>
<td>(-55 (n = 2))</td>
<td>0.15</td>
<td>0.12</td>
</tr>
<tr>
<td>(-45 (n = 4))</td>
<td>0.39 ± 0.14</td>
<td>0.61 ± 0.12†</td>
</tr>
<tr>
<td>(-55 (n = 4))</td>
<td>0.09 ± 0.04</td>
<td>0.41 ± 0.09‡</td>
</tr>
</tbody>
</table>

Explanation of data is the same as in Table 2.

* Not significantly different from single pulse block.
†‡ Significantly different from single pulse block \( (P < 0.05, P < 0.005) \).
§ Significantly different from single pulse block and 20-msec block \( (P < 0.02) \).
Figure 10. Effect of nisoldipine on voltage-dependence of Ca channel current availability: Prepulses (V_Q), 30 sec in duration, were followed by a 100 msec test pulse (V_T) to 0 mV. Graph shows normalized plot of maximum inward current measured during V_T in the absence (%) and presence of nisoldipine (200 nM A, 400 nM B) vs. V_C. Peak current in presence of nisoldipine was 87% (200 nM) and 77% (400 nM) of that recorded in control. Inset: pulse protocol and current traces in response to test pulse following 30 sec conditioning pulse to -44 mV and -74 mV, control (C), nisoldipine (N). Curve: best fit (Methods) of function (1 + exp[(V - V_h)/k])^-1 to data. Control: V_h = -32 mV, k = 4.1. Nisoldipine: V_h = -53.4 mV (200 nM), -55 mV (400 nM), k = 4.2 (200 nM), 4.4 (400 nM). 5.4 mM Sr** Tyrode’s solution + 10 µM TTX. Preparation 339-1.

is modulated by membrane potential. Block is more pronounced when Ca channel current is measured during voltage clamp pulses applied from depolarized holding potentials. Correspondingly, in preparations that are not voltage clamped, blocking activity of these compounds will be strongly influenced by cell resting potentials. This observation has important implications relevant to the use of these drugs in clinical applications and in binding studies.

Clinical Implications

Our results indicate that nisoldipine can block Ca channel current in a use-dependent manner, but that this effect becomes pronounced only under conditions of rapid stimulation. Thus, it is not surprising that previous studies have reported little or no use-dependent changes in block by this and other dihydropyridines (Bayer and Ehara, 1978; Kass, 1982; Tung and Morad, 1983). Use-dependent block by nisoldipine is important only at pulse frequencies greater than 1 Hz, whereas D600 and AQA 39 show prominent use-dependent effects at frequencies as low as 0.33 Hz.

Based on this comparison, it would appear that verapamil and other compounds such as diltiazem that show marked use-dependent block (Kanaya et al., 1983) would be suitable as antiarrhythmic agents for disorders characterized by repetitive firing of Ca-dependent action potentials. This prediction is consistent with the clinical data: verapamil and diltiazem (Rowland et al., 1983), but not nifedipine (Rowland et al., 1981), have potent antiarrhythmic activity for supraventricular tachycardias.

The dihydropyridines are more potent vasodilators than either diltiazem or verapamil, and so, for a given dose, are more effective in the treatment of angina pectoris (DePonti et al., 1979) and hypertension (Klein et al., 1983). The basis for this distinction may be due in part to the marked effect of holding potential on the blocking activity of nisoldipine, but not verapamil. Therefore, because some smooth muscle cells are characterized by depolarized resting potentials and slow repetitive activity (Horn, 1978), Ca channels in these tissues are likely to be very sensitive to low concentrations of dihydropyridines. Similar interpretations have been suggested by Tung and Morad (1983) and Schwartz and Triggle (1983).

Voltage-Dependent Block

We suggest that our results can be interpreted within the framework of the modulated receptor hypothesis proposed by Hille (1977) (also see Honeghem and Katzung, 1977) to explain local anesthetic block of Na channels in nerve and skeletal muscle. In this interpretation, binding of a drug to a receptor located within the channel is influenced by the state of the channel which is determined by membrane potential.

This model predicts that ionized drugs can only gain access to the channel-associated receptor via a
hydrophilic pathway that is available only when channels are in the open state configuration. Neutral drugs can reach the same receptor via this pathway, as well as by a hydrophobic route, through the lipid membrane surrounding the channel. These pathways as described by Hille (1977) for depolarized membrane, are shown diagramatically below. R, O, and I represent the three normal channel states: rested, open, and inactivated, respectively, and an asterisk represents drug-bound channels.

Because verapamil is almost entirely in its charged form at pH 7.4, this scheme predicts that it can only block channels by first binding to channels in the open state. If, as assumed by Hille (1977, 1978), drug-associated channels can undergo voltage- and time-dependent transitions similar to drug-free channels, then verapamil-bound channels will eventually settle into the absorbing drug-bound inactivated state (I*) during prolonged depolarization. Schematically this sequence would appear as R → O → O* → I*.

In our experiments, when membrane potential is depolarized to -45 mV in the absence of repetitive depolarizations, verapamil did not block current (Fig. 8). This result suggests that block via the open channel pathway does not occur to any measurable extent for verapamil when membrane potential is fixed (without stimulation) at the depolarized level of -45 mV. Instead, for verapamil, block develops only during repetitive pulsing to more positive voltages from this holding potential where the probability of channel opening is much greater (see also Pelzer et al., 1982).

The dihydropyridines, nisoldipine and nitrendipine, are neutral drugs at physiological pH, and therefore the model predicts that drug binding by these compounds can occur either via pathway (1) (R → O → O* → I*) and/or pathway (2) (R → I → I*), depending upon membrane voltage. Considering the results described above for verapamil, it is not likely that development of block at membrane potentials ≤ -45 mV in the absence of repetitive depolarization occurs via pathway 1. Nevertheless, in this range of voltages, holding potential alone markedly influences the amount of current blocked by these neutral drugs (Figs. 3, 4, 6, 10). These results considered together suggest that, at membrane potentials ≤ -45 mV, block by nisoldipine occurs via pathway 2. That is, in this voltage range, the drug binds to inactivated channels without the prerequisite of channels first being in the open state.

Local anesthetics alter the relationship between Na channel availability and membrane potential by causing hyperpolarizing shifts in this relationship (Hille, 1977; Khodorov, 1978; Lee et al., 1981; Bean et al., 1983). In terms of the modulated receptor scheme, this observation has suggested an interaction between drug molecules and the inactivated state of the channel. That is, drug-bound inactivated channels are very stable, and this state thus becomes the absorbing state in scheme 1 under steady state conditions (Hille, 1978). We find that nisoldipine (200 nm) causes a hyperpolarizing (about 19-mV) shift of the relationship of Ca channel availability vs. membrane potential when this relationship is measured with long (30-second) prepulses. This result is very similar to the effects of local anesthetics on the voltage-dependence of Na channel availability and is consistent with an interaction between dihydropyridines and the inactivated state of the Ca channel.

When Ca channel current is measured by applying pulses at low frequencies from negative holding potentials, nisoldipine (200 nm) causes little or no block (Fig. 1). Because virtually all drug-free channels are in the rested state at voltages negative to -60 mV (Reuter, 1979; Reuter et al., 1982; Kass and Sanguinetti, in press), this result suggests that, at this drug concentration, there is weak binding of nisoldipine to channels that are in the rested state (R → R*).

Although our results suggest that, in the steady state, voltage-dependent modulation of block by nisoldipine is primarily due to a strong affinity of this drug for the inactivated state, we also tested for and found evidence of open channel block by nisoldipine. Support for the interpretation of open channel block comes from the observed increase in the apparent rate of inactivation during the first depolarization applied in the presence of drug (Fig. 5; Table 1). This confirms the results reported by Lee and Tsien (1983) in enzymatically dispersed ventricular muscle cells.

As discussed above, our results suggest that, in the absence of repetitive pulsing from holding potentials ≤ -45 mV, open channel block is relatively unimportant to the actions of nisoldipine. But, in the presence of repetitive depolarization, our data suggest that open channel block can occur by this drug and contribute to the overall reduction of Ca channel current under these conditions (Figs. 6 and 7). The pulse train protocols of our experiments minimized possible contributions of open channel block during repetitive activity. More rapid pulsing would have resulted in enhanced inhibition of current, perhaps due to more channel block. Quantitative determination of the contribution of open channel block to the actions of these drugs during repetitive depolarization may require additional experimental procedures such as single channel recordings or rapid photoconversion procedures (Gurney et al., 1984).

Finally, additional support for a modulated recep-
tor interpretation of our data comes from the experiments with nicardipine, where use-dependent block showed behavior intermediate to the other drugs as predicted by the ionization constant of this compound (see Pelzer et al., 1982, for discussion of two verapamil derivatives). The distinction is important because evidence from binding data suggests that verapamil and the dihydropyridine derivatives act at different receptor sites (Janis and Triggle, 1983).

Comparison to Previous Studies

Previous investigations of the actions of nifedipine suggested that some dihydropyridines did not alter the voltage-dependence of Ca channel availability (Bayer and Ehara, 1978; Bayer et al., 1982) or change the kinetics of recovery from inactivation (Kohlhardt and Fleckenstein, 1977; Bayer et al., 1982), although Lee et al. (1983) reported that nifedipine promotes a slow phase of recovery from inactivation.

In some of these previous experiments, prepulses (500 msec) were used that were too brief to allow the effects of the drug to develop, and recovery from block was measured at holding potentials (−40 to −50 mV) where the time constant for recovery from inactivation of drug-free channels is very slow (Kass and Scheuer, 1982; Kass and Sanguinetti, in press) and resembles the time course of removal of block by the drugs. The amount of slow inactivation of Ca channel current observed in the absence of drug at voltages near −45 mV is variable (0–30% decrease in peak inward current) in the calf Purkinje fiber. This variation is evident upon comparison of the control records in Figures 3 and 10.

A previous investigation of the influence of membrane potential on Ca channel current block by nisoldipine (Kass, 1982) used voltage protocols and drug concentrations that failed to resolve the voltage-dependent changes in block reported in the present study. In the previous study, most experiments were carried out at drug concentrations of 10 μM where block by nisoldipine, but not D600, was voltage-independent. Furthermore, voltage clamp protocols imposed at least a 2-second pause at depolarized holding potentials before repetitive test depolarizations were applied. Therefore, block that developed during this pause in low concentrations of nisoldipine was not detected.

Binding Studies

Several groups have reported the results of binding studies using 3H-dihydropyridine compounds in cardiac, smooth, and skeletal muscle preparations (reviewed by Janis and Triggle, 1983). The first and most extensive binding studies have been carried out in intestinal smooth muscle. This work described the binding as stereospecific, high affinity (K_D = 0.1–1.0 X 10^-9 M) rapid, and reversible (Janis and Triggle, 1983).

A key test to show that ligand binding is occurring at the pharmacologically relevant site is to demonstrate agreement between binding data and inhibition of the biological response. This has been satisfactorily demonstrated in the case of smooth muscle, where inhibition of contractile activity occurs over a concentration range similar to that reported for the binding constant of dihydropyridines (Janis and Triggle, 1983). However, the data for cardiac muscle do not correlate as well. In this case, the K_D for binding is 2–3 orders of magnitude lower than the drug concentrations reported to produce negative inotropic effects (Ferry and Glossman, 1982; Bolger et al., 1983) or block Ca channel current (Kass, 1982; Lee and Tsien, 1983).

We can estimate dissociation constants for nisoldipine binding to the resting and inactivated states in our experiments by using an approach described by Bean et al. (1983) in the analysis of state-dependent binding by lidocaine to cardiac Na channels. We first assume that the data presented in Figure 10 represent binding to channels only in the rested or inactivated state. Next, we use the data for currents measured from the −80 mV holding potential. Assuming one-to-one binding, we compute the rested state dissociation constant (k_R) from the peak current present (relative to control) in 200 nM (87%) and 400 nM nisoldipine (77%). Computations from the current remaining at both drug concentrations predict a value of k_R = 1340 nM.

According to Bean et al. (1983), the shift in the midpoint of the steady state availability curve (∆V_h) is related to the nisoldipine concentration (N) and dissociation constants for binding to inactivated channels (k_i) and rested state channels (k_R) as follows:

\[-\Delta V_h = k \ln[(1 + (N)/k_i)/(1 + (N)/k_R)] \]

(2)

where k is the slope factor determined from the control steady state availability curve (k = 4.1 in Fig. 10). Plugging the shift (∆V_h) of −21.4 mV caused by 200 nM nisoldipine in Figure 10, along with the above value for k_R into Equation 2, gives a value of 1.0 nM for k_i.

An additional check on this computation is provided by the 400 nM data of Figure 10. Using the value of k_i determined above, along with a 400 nM nisoldipine concentration in Equation 2 predicts a 23.7-mV hyperpolarizing shift of the availability curve. This prediction is in excellent agreement with the data of Figure 10 which show a −23-mV hyperpolarizing shift in the presence of 400 nM nisoldipine.

Thus, our data suggest that binding is approximately 1000 times stronger to inactivated channels than to resting channels. This three order of magnitude difference in binding affinities is very close to the differences between previously reported cardiac electrophysiological and binding data.

The results of the present study show that membrane potential must be considered when comparing
any of these data. Binding studies that are carried out in membranes of depolarized cells or obtained from membrane fragments are carried out under conditions in which transmembrane potential gradients are minimized and drug-blocking activity is most pronounced. Vascular smooth muscle preparations, in which resting potentials may be more positive than $-60 \text{ mV}$ (Horn, 1978), more closely resemble these conditions than cardiac ventricular cells, and, thus, would be expected to provide concentration-response results similar to the binding studies, especially when contractions are elicited by exposure to depolarizing high potassium solutions. Recent studies of changes in binding to isolated cardiac ventricular cells in solutions containing different concentrations of KC1 support this view (Green et al., 1983).

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