# Influence of $pH_o$ on Calcium Channel Block by Amlodipine, a Charged Dihydropyridine Compound

## *Implications for Location of the Dihydropyridine Receptor*

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ABSTRACT We have investigated the modulation of L-type calcium channel currents in isolated ventricular cells by the dihydropyridine derivative amlodipine, a weak base with a $pK_a$ of 8.6. Under conditions that favor neutral drug molecules, amlodipine block resembles other, previously described, neutral dihydropyridine derivatives: block is more pronounced at depolarized voltages, repetitive pulsing is not needed to promote block, and recovery is complete at hyperpolarized voltages. When the drug is ionized, depolarized voltages still enhance block, however, the time course is slow and speeded by repetitive pulses that open channels. Recovery from block by ionized drug molecules is very slow and incomplete, but can be rapidly modified by changes in external hydrogen ion concentration. We conclude from these observations that the degree of ionization of the drug molecule can affect access to the dihydropyridine receptor and that external protons can interact with the drug-receptor complex even if channels are blocked and closed. These observations place limitations on the location of this receptor in the ventricular cell membrane.

## INTRODUCTION

The importance of dihydropyridine (DHP) derivatives as probes of L-type calcium channels is now well-established (Triggle and Janis, 1984; Glossman and Ferry, 1985; Triggle and Venter, 1987). DHP compounds bind with high affinity to receptors associated with the calcium channel protein and cause gating of these channels to change (Bean, 1984; Hess et al., 1984; Sanguinetti and Kass, 1984; Kokubun et al., 1987).

The DHP receptor, which is closely linked to the L-type calcium channel, has been purified from rabbit skeletal muscle (Borsotto et al., 1984; Curtis and Catter-

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Volume 93 June 1989 1109–1127

all, 1984; Flockerzi et al., 1986) and its structure has been determined (Tanabe et al., 1987). The receptor polypeptide has homology with voltage-dependent sodium channels both in amino acid sequence and proposed transmembrane topology. Because of the structural similarities between the sodium channel and the purified DHP receptor it is important to compare functional similarities between sodium and (L-type) calcium channels.

The investigation of sodium channel block by neutral and charged local anesthetics has provided a great deal of information about the location of the local anesthetic receptor in the sodium channel as well as the structure of the channel itself (Hille, 1977*a*, *b*; Hondeghem and Katzung, 1977; Schwarz et al., 1977). The experimental strategy of these investigations was to control the contribution of neutral or ionized drug molecules to channel block by varying the external pH. The results of these studies have been essential in postulating molecular models of the sodium channel (see Hille, 1984; Begenisich, 1987).

Previous investigations of the functional consequences of DHP binding have used compounds such as nifedipine, nisoldipine, nitrendipine, and PN 200–110, which are neutral molecules at physiological pH. In the present study, we investigated modulation of calcium channel currents by amlodipine, a DHP derivative that competes for nitrendipine binding sites (Burges et al., 1985) but is mostly charged at physiological pH and neutral in more alkaline solutions. The purpose of this work was to vary the ratio of charged to neutral molecules of this drug to provide information about the location of the DHP receptor in cardiac ventricular cell membranes. Our results show that the drug-bound receptor is accessible to external hydrogen ions suggesting either a direct aqueous pathway to a receptor site within the channel or a receptor location near the outer face of the sarcolemmal membrane.

## METHODS

Single ventricular myocytes were isolated from either ventricle of adult guinea pigs using a method similar to that of Mitra and Morad (1985) which has been previously described (Arena and Kass, 1988).

Recording methods were as described by Hamill et al. (1981) for the whole-cell configuration. Patch pipettes were made from Gold Seal Accu-fill 90 Micropets (Clay Adams, Inc., Parsippany, NJ). The resistance of the pipettes was typically 1–3 mΩ when filled with 140 mM CsCl. Series resistance compensation was used in all experiments and was adjusted to give the fastest possible capacity transients without producing ringing. Data were sampled once every 0.3 ms and filtered at 1–2 kHz with an 8-pole Bessel filter (Frequency Devices, Haverhill, MA).

### *Solution and Drugs*

Solutions and buffers used in these experiments are described in detail in Krafte and Kass (1988). Briefly, solutions were chosen to eliminate K channel currents. Thus, the standard pipette solution contained in millimolar: 100 CsCl, 40 CsOH, 2 $MgCl_2$, 1 $CaCl_2$, 11 EGTA, 2–5 $K_2ATP$, 10 HEPES (pH 7.4). The standard bath solution contained in millimolar: 132 NaCl, 4.8 CsCl, 1.2 $MgCl_2$, 5 glucose, 5 HEPES (pH 7.4). NaCl was replaced by Tris-Cl in order to eliminate currents through Na channels in some experiments. In experiments in which $pH_o$ was different from 7.4, one of the following buffers where appropriate was used:

CAPS (3-[cyclohexylamino]-1-propane sulfonic acid) ($pK_a$ 10.1) or MES (morpholine ethane sulfonic acid) ($pK_a$ 6.15). These buffers were purchased from Sigma Chemical Co., St. Louis, MO. Divalent cations were added as $Cl^-$ salts as indicated in each experiment.

Hydrogen ion concentration of test solutions was adjusted to alter the net charge of drug molecules. For a drug molecule with an acid dissociation constant $K_a$, the fraction of drug in the neutral form (N) is given by the bimolecular formula:

$$N = K_a/(K_a + [H]_o). \quad (1)$$

Nisoldipine, a gift from Miles Laboratories, New Haven, CT, was dissolved in polyethylene glycol 400 (PEG) to make a concentrated stock, and diluted in the bath to the final concentration. PEG at the concentration used (>1,000× dilution) has been shown to have no effects of its own on Ca channel currents (Kass, 1982). Amlodipine (see Fig. 1 for structure), a gift from Pfizer Central Research, Sandwich, UK, was dissolved in water as a concentrated stock solution. Previous investigations have shown that displacement of nitrendipine binding (Burges et al., 1985) and inhibition of Ca-dependent contractions (Burges et al., 1987) can be measured at nanomolar amlodipine concentrations, but these effects take several hours to develop. Furthermore, the same studies have shown that onset times of amlodipine effects are greatly reduced by using micromolar drug concentrations. Thus, because of the realistic time limitations of our experiments, we chose to use micromolar amlodipine concentrations

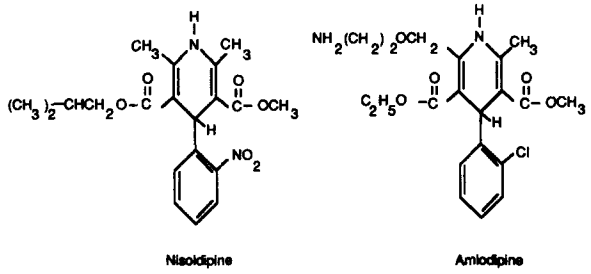


FIGURE 1. Structures of the two DHP compounds used in this study: nisolidpine (*left*) and amlodipine (*right*). The $pK_a$ for nisoldipine has been estimated to be <3.5 (Hugenholtz and Meyer, 1987). The $pK_a$ for amlodipine is 8.6.

in this study. At these concentrations, the voltage-dependent effects of amlodipine were measurable within 2 min after exposing cells to amlodipine-containing solutions.

### *Voltage Protocols*

In experiments that required recording currents from potentials negative to −60 mV, 50–100-ms prepulses were applied to −40 mV to inactivate sodium channel and T-type calcium channel currents (Bean, 1985; Marchetti and Brown, 1988). Thus in this paper current referred to as Ca channel current ($I_{Ca}$) corresponds to L-type Ca channel current according to the terminology suggested by Nilius et al. (1985). Sodium channels were also blocked by 10–50 μM tetrodotoxin (TTX) (Behring Diagnostics, La Jolla, CA) and by replacement of NaCl by Tris-Cl in some experiments.

Voltage protocols designed to measure the kinetics of drug onset and recovery were similar to those previously described by Sanguinetti and Kass (1984). Briefly, after a pulse-free period at a fixed holding potential, the holding potential is changed and repetitive pulses are applied to a voltage that opens calcium channels. This is referred to as a pulse train protocol. Holding potentials, pulse durations, and pulse rates are defined in each experiment and are chosen to either cause block or recovery from block during the pulse train. The duration of the repetitive pulses is varied and specified with each experiment. Block that develops in response to these protocols is caused by the change in holding potential and the repetitive

voltage pulses. "Single-pulse" protocols consisted of brief pulses applied very infrequently after a change in holding potential. Block that develops in response to a single-pulse protocol is primarily a function of the change in holding potential because test pulses are applied so infrequently. In some experiments designed to study recovery from inactivation or block, long depolarizing conditioning pulses precede pulse trains which are then applied from a negative holding potential. The first pulse of each sequence is applied just before the change in holding potential. Subsequent pulses are applied from the new holding potential. These protocols are illustrated schematically in the insets of Fig. 2.

### *DHP Receptor: Agonism and Antagonism*

Most DHP compounds are capable of causing enhancement or block of $I_{Ca}$ depending on cell membrane potential (Hess et al., 1984; Sanguinetti et al., 1986; Kass, 1987). Binding and electrical data have provided conflicting evidence that these effects are due to interactions with more than one binding site (Williams et al., 1985; Brown et al., 1986; Kokubun et al., 1986; Wei et al., 1986; Hamilton, et al., 1987). In this study we observed agonistic activity of amlodipine that was somewhat variable from cell to cell, but we did not attempt to test for separate receptors responsible for agonism or block. The experiments were focused on changes in accessibility to a receptor responsible for blocking or inhibitory activity of the drug. In the manuscript for simplicity, this receptor is referred to as the DHP receptor. We, however, do not imply that a separate receptor responsible for agonism does not exist.

### *Curve Fitting and Statistical Procedures*

Experimental data were fitted with functions of one or two exponentials plus an arbitrary baseline using procedures previously described (see Sanguinetti and Kass, 1984). Data between two groups were compared using an unpaired $t$ test with a significance level of $P < 0.05$ (Rosner, 1982). Data averaged from several experiments are presented as mean ± SEM.

## RESULTS

### *Nisoldipine Block of $I_{Ca}$ Is Not Affected by $pH_o$*

To determine that changes in drug activity are related to the relative fractions of charged or uncharged molecules, it is necessary to establish the independence of the drug receptor from the alterations in external pH.

Nisoldipine is a well-characterized DHP compound with a $pK_a$ of <3.5 (Hugenholtz and Meyer, 1987) that is virtually entirely neutral over a $pH_o$ range of 6.0–10. Fig. 2 shows that the onset of and recovery from block by nisoldipine is not affected as $pH_o$ is changed from 7.4 to 10.0. The inhibition of $I_{Ca}$ in this experiment was caused by the neutral form of the drug molecule, and because the fraction of neutral drug was virtually constant, the results show that interactions of neutral drug molecules with the DHP receptor are not modified by the $pH_o$ changes we imposed.

Fig. 2 *A* shows the results of experiments designed to promote nisoldipine block of $I_{Ca}$ in solutions of pH 7.4 and 10. Train protocols in which brief pulses were applied repetitively from −40 mV (Methods) were imposed in the absence and presence of drug. It is clear that drug-induced reduction of current is not affected by the change in external pH.

Recovery of current after drug block had developed is shown in Fig. 2 *B* under conditions of $pH_o$ 10.0 and 7.4. In the presence of drug, recovery of current is characterized by a slow component that is much more prominent than in control conditions. This component is consistent with the relief of drug-blocked channels at the negative holding potential (see also Kass and Sanguinetti, 1984). Slow recovery from drug-induced block is evident in both $pH_o$ 7.4 and 10.0, and the time constant for the drug-induced slow component of recovery from block was 30 s in both solu-

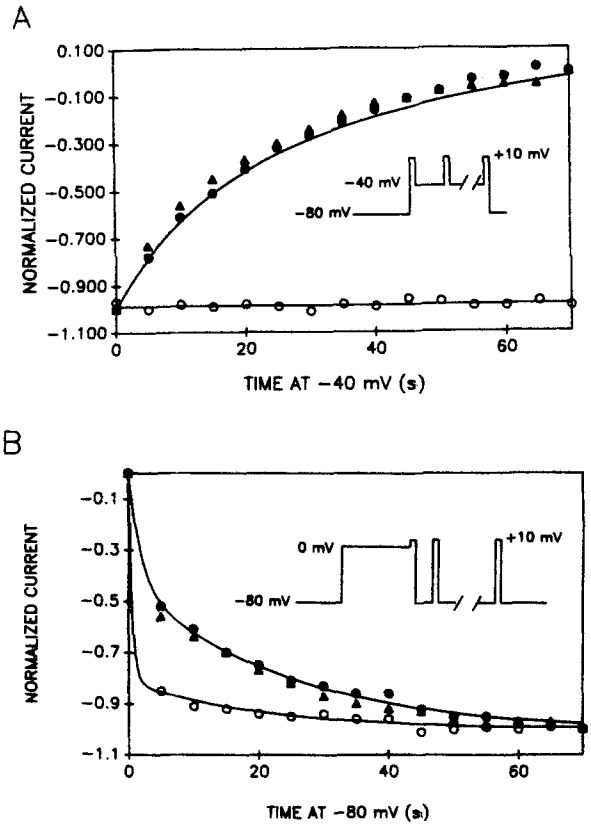


FIGURE 2. Influence of external pH on the development of and recovery from block by nisoldipine. Control data were measured in pH 7.4 (○). Data were then obtained in the presence of 50 nM nisoldipine in pH 7.4 (▲) and finally in $pH_o$ 10.0 (●). (*A*) Onset of block. Pulse train protocol (*inset*, and Methods) designed to promote block caused no change in control currents but inhibited currents in the presence of nisoldipine in both pH 7.4 and 10.0. Currents are plotted against the time after the change in holding potential. The curve through the control data is intended only as a visual aid. The smooth curve through the nisoldipine data is a function of two exponentials fitted to the $pH_o$ 10.0 data: $\tau$'s are 5 and 30 s. Pulse rate during the train was 0.2 Hz and the test pulse width was 40 ms. (*B*) Recovery from block. Currents were measured from a −80-mV holding potential after a 20-s conditioning pulse to 0 mV (*inset*). Brief (20 ms) pulses to +10 mV were applied at 0.5 Hz. Currents are plotted against time after change from 0-mV conditioning voltage to −80-mV holding potential. The time constants for the exponential curves are 0.6 and 29 s (control), 2 and 31 s (drug). 2 mM Ca. Cell 7J81.

tions. We conclude from these experiments that external pH does not alter the DHP receptor.

### *Amlodipine: Weak Blocking Activity for Rested Channels*

Fig. 3 shows that amlodipine resembles other DHP compounds in that $I_{Ca}$ is not blocked if the resting membrane potential is sufficiently negative. In this experi-

ment, which was carried out at $pH_o$ 7.4, currents were measured under control conditions from a −80-mV holding potential. The cell was then exposed to the drug. During the solution change and for the next 5 min the membrane was held at −80 mV without application of test pulses. Then, while the holding potential was maintained at −80 mV, currents were measured in the presence of amlodipine and found to be little changed from control values. Similar results (not shown) were also obtained in solutions buffered to $pH_o$ 10.0. Under similar conditions, we often detected amlodipine-induced enhancement of $I_{Ca}$, consistent with agonist-like activity of the drug (data not shown, but see Fig. 4). On average, currents measured from −80-mV holding potentials were increased 19.7 ± 6.6% ($n$ = 13) at $pH_o$ 7.4 and 10.4 ± 5.9% ($n$ = 7) at $pH_o$ 10. These results show that amlodipine, like other

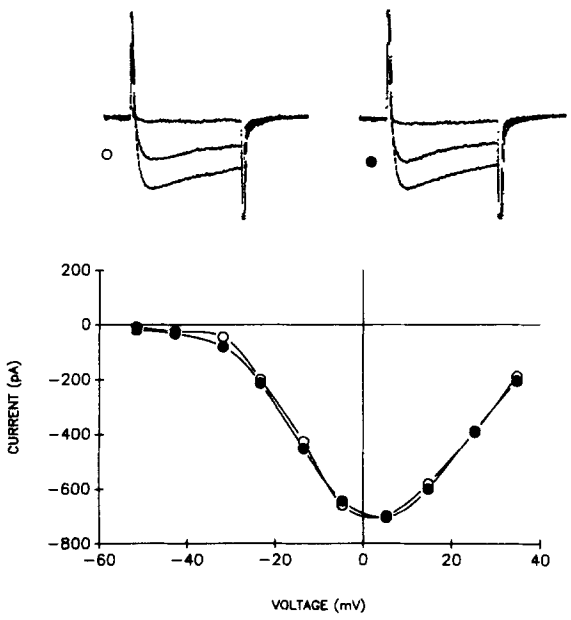


FIGURE 3. Amlodipine does not block currents measured from −80 mV holding potential. Currents measured in response to 40-ms voltage pulses applied from a −80-mV holding potential are plotted against pulse voltage. Pulses were applied at 0.5 Hz and currents were measured in the absence (o) and presence (•) of 3 μM amlodipine. The cell was exposed to the drug at a −80-mV holding potential and held without stimulation for 5 min in the presence of drug before currents were measured. The insert shows current traces in response to pulses to −30, −15, and +10 mV in the absence (*left*) and presence (*right*) of amlodipine. 2 mM Ca. $pH_o$ = 7.4. Cell 9304.

DHP derivatives, is not a potent blocker of channels in the rested state, but can enhance currents measured from negative holding potentials. Furthermore, the results show that the agonistic activity of the drug molecule does not have a dependence on $pH_o$.

*Amlodipine: Drug Block by Neutral Molecules Is Similar to Nisolidpine*

We next designed conditions to promote amlodipine block of $I_{Ca}$ and focused first on interactions of the neutral form of the drug with the DHP receptor. The $pK_a$ for amlodipine is 8.6, and thus, according to Eq. 1, the drug molecules are predominantly neutral (96%) in solutions buffered to pH 10. Fig. 4 illustrates the develop-

ment of block by neutral amlodipine caused by changes in membrane potential in solutions buffered to $pH_o$ 10.

The experiments summarized in Fig. 4 were designed to permit comparison of block by the neutral amlodipine molecule to block by nisoldipine. Development of block was measured during application of a train of brief pulses (Methods) and, for comparison, with infrequent application of single pulses.

Membrane potential was held at −80 mV without pulsing as the cell was exposed to amlodipine, to minimize possible drug-induced block. After the extracellular solution change was complete, the two protocols described above were repeated. The single-pulse protocol was applied first, and, as can be seen in the figure, inhibi-

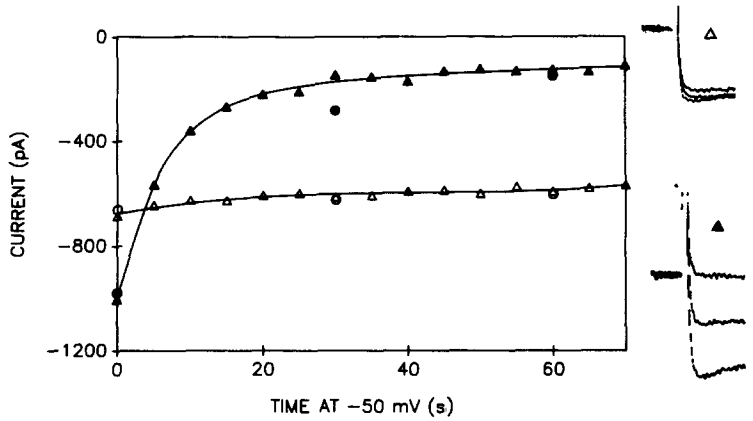


FIGURE 4. Onset of amlodipine block in $pH_o$ 10.0: influence of voltage protocol. Currents were measured in response to single-pulse and pulse-train protocols in the absence (*open symbols*) and presence (*filled symbols*) of amlodipine (3 μM) in $pH_o$ 10.0. The pulse-train protocol (*triangles*) was similar to that described in Fig. 2 *A*, except that the holding potential was changed from −80 to −50 mV. In the single-pulse protocol (*circles*) pulses were applied once every 30 s. Currents are plotted against time after change from −80 mV to the −50-mV holding potential. The smooth curve through the control data is drawn through the points as a visual aid. The curve through the currents measured in drug is a function of two exponentials with time constants 6 and 50 s. The cell was held at −80 mV without stimulation for 2 min between successive runs to allow from recovery from block and inactivation. The inset shows current trances from the train protocols in control (*open symbols*) and drug (*filled symbols*) solutions measured at 0, 5, and 45 s after the change in holding potential.

tion of $I_{Ca}$ developed quickly when the conditioning voltage (−50 mV) was imposed. This procedure emphasizes changes in drug activity due to the depolarized conditioning voltage because pulses that cause channels to open are applied so infrequently. Under these conditions, 90% block was measured within 60 s. The currents in response to the first pulse of this protocol were actually larger than control currents, indicating amlodipine-induced agonism at the −80-mV holding potential.

After the single-pulse protocol was used to generate block, the membrane was held at −80 mV for 2 min without pulsing and a second train protocol was resumed. Block induced by the single-pulse experiments was completely relieved

at −80 mV during this period. It redeveloped with approximately the same time course during a subsequent train of brief (20 ms) pulses applied once every 5 s from the −50-mV conditioning voltage. Application of a train of 200-ms pulses slightly speeded the development of block during the first four pulses, but did not change the total fraction of current blocked (not shown) at the end of the pulse train.

Fig. 5 illustrates a representative experiment designed to probe the time course of $I_{Ca}$ recovery from block by neutral amlodipine. It shows the recovery from a 20-s conditioning prepulse to 0 mV in the absence and presence of drug in solutions buffered to $pH_o$ 10. Recovery of current after the conditioning pulse follows a time course described by a function of two exponentials. The fast component ($\tau$ = 1 s) is most likely due to recovery of drug-free channels, and the slow component ($\tau$ = 10 s) reflects recovery from drug-blocked channels at the negative voltage.

All of these observations resemble voltage-dependent effects of previously investigated neutral DHP derivatives (Bean, 1984; Sanguinetti and Kass, 1984). Thus we

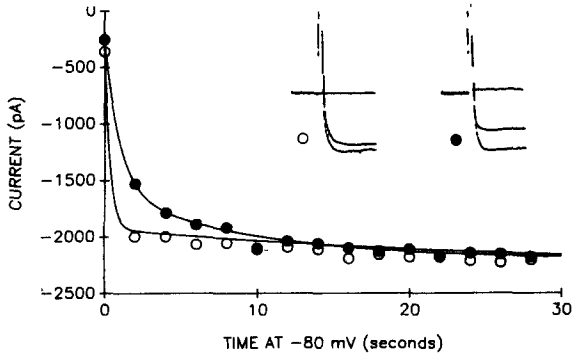


FIGURE 5. Recovery from amlodipine block in $pH_o$ 10.0. Currents were measured with the similar protocol discussed in Fig. 2 *B* in the absence (○) and presence (●) of 3 μM amlodipine. Currents were measured in response to 40-ms pulses to 0 mV and plotted against time after termination of the 20-s conditioning pulse to 0 mV. The smooth curves are exponential functions with the following time constants: 1 s (control); 1, 9 s (drug). The inset shows currents traces in the absence (*left*) and presence (*right*) of drug measured 0, 2, and 28 s after returning to −80 mV. 2 mM Ba. Cell 7281.

conclude that the voltage dependence of amlodipine recorded in solutions buffered to $pH_o$ 10 is the same as that of nisoldipine and other neutral DHP compounds. The neutral drug molecule inhibits channels in depolarized membranes more potently than in polarized membranes and channel openings are not a prerequisite for drug action. Furthermore, block is reversible upon repolarization to voltages negative to −70 mV.

### *Amlodipine: Effects of the Charged Molecule*

*Repetitive depolarization enhances block.* The next set of experiments was designed to investigate block of $I_{Ca}$ by the charged amlodipine molecule. The majority of these experiments were carried out at $pH_o$ 7.4 where 94% of amlodipine molecules are ionized (Eq. 1) and most of the drug activity is expected to be due to the charged drug form. Extensive experiments in more acidic solutions (i.e., $pH_o$ 6.0) were avoided because currents were not sufficiently stable for the long periods required for most of the tests we imposed.

Fig. 6 illustrates the development of block by amlodipine in $pH_o$ 7.4 with two different voltage protocols. The results presented can be compared to those shown in Fig. 4 for neutral drug molecules. Fig. 6 shows that voltage-dependent block develops slowly in $pH_o$ 7.4 in response to a single-pulse protocol that emphasizes the effects of holding potential on drug block. In this experiment, the slow time course of block induced by the change in holding potential ($\tau$ = 103 s) was considerably speeded by the simultaneous application of a pulse train protocol in which repetitive

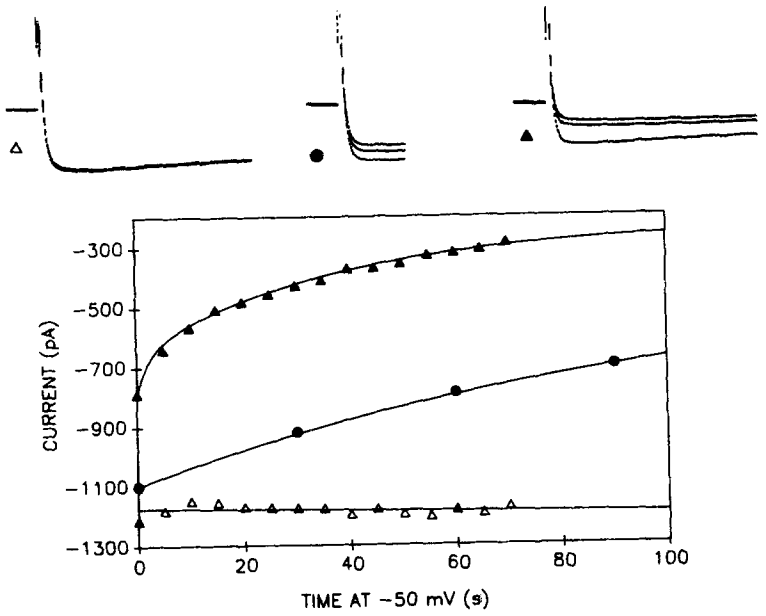


FIGURE 6. Development of amlodipine block in $pH_o$ 7.4: influence of holding potential. A pulse-train protocol similar to that described in Fig. 2 *A* was applied in the absence (△) and presence of 3 μM amlodipine (▲). The holding potential was changed from −80 to −50 mV and 200-ms pulses were applied at 0.2 Hz. In the presence of drug a single-pulse protocol (●) was also applied to test for the role of the holding potential in amlodipine block. Here 20-ms pulses were applied once every 30 s. In the presence of drug, the single-pulse protocol was applied first, followed by the train protocol. The cell was held for 3 min without stimulation at the −80-mV holding between runs. Measured currents are plotted against the time after changing holding potential from −80 to −50 mV. The line through control data is intended only as a visual aid. The curves in the presence of drug are fitted exponential functions with the following time constants: pulse train, 2.5, 38 s; single pulse, 100 s. The insets show currents measured 0, 30, and 60 s after changing the holding potential to −50 mV. The first 66 ms of the 200-ms pulses are shown.

pulses (200-ms duration) are applied along with the change in holding potential. Under these conditions, the time course of current was described by a two-exponential process with time constants 2.5 and 37 s.

Another important point illustrated by the data of Fig. 6 is the fact that recovery from block in $pH_o$ 7.4 was usually incomplete. In this experiment, the membrane

potential was held at −80 mV for 3 min after the single protocol and before imposing the second pulse train of 200-ms pulses. Despite the long period without stimulation at a negative voltage, there was little recovery of block that had developed during the first pulse protocol. We consistently found a pattern of incomplete recovery from block in solutions that favor ionized drug molecules. In 16 experiments, the mean fraction of current recovered at −80 mV was 0.35 ± 0.06. This is

TABLE I

*Influence of $pH_o$ on Recovery from Drug Block: Fraction Recovered and Kinetics of Slow Component to Recovery*

| Experiment | pH | Time constant | Fraction recovered |
|---|---|---|---|
| 723-1 | 7.4 | 40 | 0.47 |
| 716-1 | 7.4 | 63 | 0.3 |
| 3F83 | 7.4 | 185 | 0.56 |
| 31M81 | 7.4 | 103 | 0.5 |
| 24M81 | 7.4 | 168 | 0.85 |
| 6A81 | 7.4 | 38 | 0.6 |
| 32687-1 | 7.4 | | 0.5 |
| 31887-1 | 7.4 | | 0.38 |
| 11487-1 | 7.4 | | 0.56 |
| 1216-2(87) | 7.4 | | 0.10 |
| 1215-3(87) | 7.4 | | 0.08 |
| 1215-5(87) | 7.4 | | 0.11 |
| 930-4(87) | 7.4 | | 0 |
| B8330 | 7.4 | 100 | 0.29 |
| A8427 | 7.4 | | 0.18 |
| B8427 | 7.4 | | 0.17 |
| Mean | | 99.57 ± 22 ($n$ = 7) | 0.35 ± 0.06 ($n$ = 16) |
| 728-1 | 10 | 9.2 | 1.0 |
| 729-3 | 10 | 8.4 | 1.0 |
| 721-1 | 10 | 37 | 0.9 |
| 721-3 | 10 | 5 | 1.0 |
| 721-2 | 10 | 3 | 0.91 |
| 28J8-2 | 10 | | 0.68 |
| 915-1(88) | 10 | 9 | 0.67 |
| 915-2(88) | 10 | 9 | 0.74 |
| 929-1(87) | 10 | 4 | 1.0 |
| 20J8-4 | 10 | 5 | 1.0 |
| Mean | | 9.96 ± 3.5 ($n$ = 9) | 0.89 ± 0.04 ($n$ = 10) |

in marked contrast to the recovery from block under conditions that favor neutral drug molecules. The mean fraction of current recovered in $pH_o$ 10 was 0.89 ± 0.04 ($n$ = 10) (see Table I for summary).

*Recovery from block is slow in $pH_o$ 7.4.* Fig. 7 shows that, for recoverable current in $pH_o$ 7.4, the recovery from block at −80 mV is very slow. Because repetitive pulses that open channels are more effective at promoting block than conditioning pulses alone in pH 7.4 (Fig. 6), a pulse-train protocol was first used to induce chan-

nel block in this experiment at a −40-mV conditioning voltage. There was no slowly developing inactivation of currents using this protocol in the absence of drug (not shown). The cell was then returned to −80 mV to monitor relief of block at a −80-mV holding potential. At this voltage records were taken once every 20 s for a period up to 5 min to monitor very slow changes in currents.

Recovery from block was significantly slower in $pH_o$ 7.4 than in $pH_o$ 10. In the experiment of Fig. 7, the drug-induced component of recovery was best described by a 103-s time constant. This is near the mean of 100 s that we measured in six similar experiments in $pH_o$ 7.4 (Table I). In contrast, the average recovery time constant in $pH_o$ 10 was 10 s (Table I and Fig. 5).

*The drug-receptor complex is sensitive to $pH_o$.* Our results show that there is a striking difference in the recovery from amlodipine block when external pH is changed. The most likely explanation for this is that protons in the extracellular solution can affect drug molecules that are bound to their receptors. Experiments designed to test for this possibility more directly are shown in the next two figures.

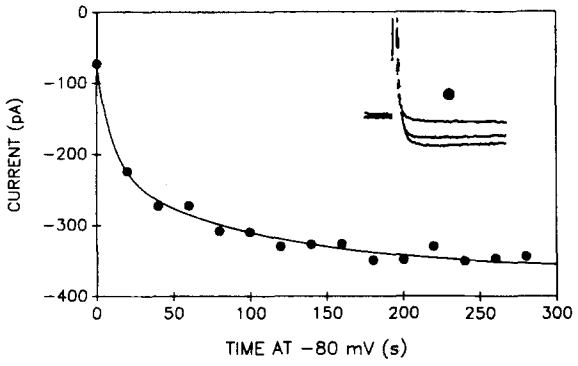


FIGURE 7. Slow recovery from amlodipine block in $pH_o$ 7.4. After development of block, the cell was returned to a −80-mV holding potential and currents were measured in response to 20-ms pulses applied to +10 mV at 0.05 Hz. Measured current is plotted against time after returning to −80-mV holding potential. The smooth curve is a function of two exponentials determined as described in Methods: $\tau$'s, 11 and 103 s. Note however, because current amplitude was sampled once every 20 s, the first component is only an approximation and probably reflects more than one process. 5 mM Ba. Cell 31M8.

Fig. 8 shows an experiment in which amlodipine block of $I_{Ca}$ was induced in $pH_o$ 7.4. In this solution, the membrane potential was returned to −80 mV, and currents only partially recovered after 75 s at this potential. The pH of the extracellular solution was then changed to 10.0, and block of the channels was rapidly relieved. In this experiment, currents were measured from a −80-mV holding potential as the external solution was changed and recovery of currents was complete within 50 s of the solution change. This is roughly the time required to change the solution in the experimental chamber.

Fig. 9 is an example of a similar experiment carried out over a more extreme range of extracellular pH. Here block onset was measured in $pH_o$ 6.0, and recoveries were compared in $pH_o$ 6.0 and 10.0. In $pH_o$ 6.0, <0.5% of the drug molecules are neutral (Eq. 1). Block developed very slowly in this experiment despite the fact that currents were measured in response to 200-ms pulses. After one-third of the available current was blocked, possible recovery from block was assayed by applying

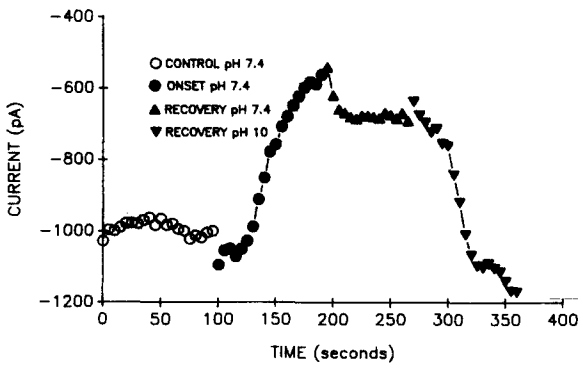


FIGURE 8. Influence of $pH_o$ on recovery from amlodipine block: $pH_o$ 7.4 and 10.0. Membrane currents were measured using voltage protocols that favor development of and recovery from block in solutions buffered to $pH_o$ 7.4 and 10.0. Control currents were measured in the absence of drug in response to 200-ms pulses applied from a −40-mV holding potential (○) in $pH_o$ 7.4. The cell was then exposed to amlodipine (3 μM) at $pH_o$ 7.4, and currents were measured in response to the same voltage protocol (●) during the solution change. After development of block, the holding potential was changed to −80 mV and currents were measured in response to 20-ms pulses with $pH_o$ still at 7.4. These currents (▲) indicate little recovery from block. With the holding potential fixed at −80 mV, $pH_o$ was then changed to 10.0, and currents were measured in response to 20-ms pulses recorded during the solution change (▼). Recovery was rapidly apparent. Pulse rate was 0.2 Hz and pulse voltage was +10 mV in all runs. Currents are plotted against time after application of first pulse in control. 2 mM Ca. Cell 12162.

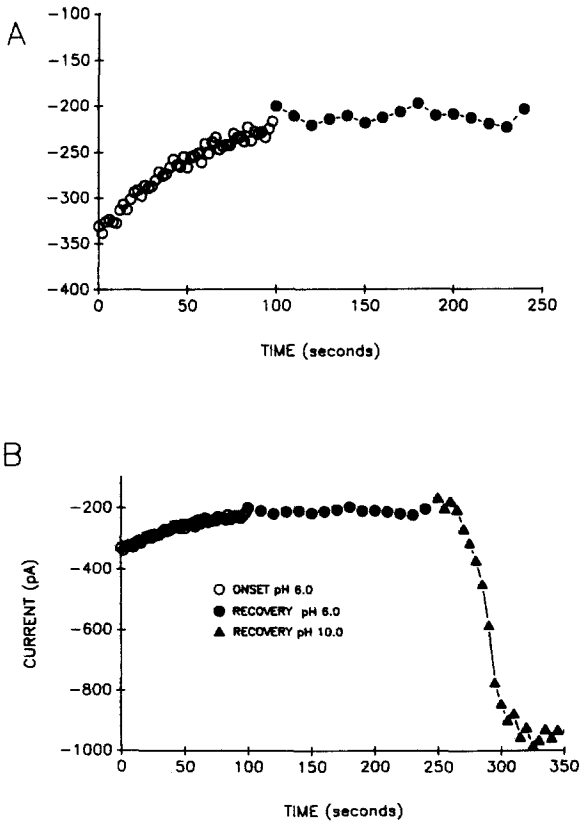


FIGURE 9. Influence of $pH_o$ on block and recovery: $pH_o$ 6.0 and 10.0. (*A*) $pH_o$ 6.0. With the holding potential fixed at −80 mV, block was induced by application of 200-ms pulses to 0 mV at 0.5 Hz (○) and possible recovery was monitored by applying 20-ms pulses to 0 mV at 0.1 Hz (●). No recovery from block was measured. (*B*) Relief of block in $pH_o$ 10.0. (○) and (●) are same as in *A*. With the holding potential fixed at −80 mV, currents were measured in response to 20-ms pulses applied to 0 mV at 0.2 Hz during a solution change to $pH_o$ 10.0 (▲). 2 mM Ca. Cell 20J84.

brief (20 ms) pulses from −80 mV once every 10 s for 2.5 min, but no recovery was detected. External pH was then changed to 10, and the membrane potential was kept at −80 mV. Current amplitude increased as fast as the solution was changed.

We observed similar effects of external pH on recovery from drug block in a total of eight cells. In two of these cells, current was completely blocked by amlodipine in $pH_o$ 7.4, and full recovery was obtained at −80 mV upon changing to $pH_o$ 10.0. In the remaining six cells, incomplete block was induced in $pH_o$ 7.4 (as in Figs. 6 and 7) and the mean increase in available current when $pH_o$ was changed from pH 7.4 to 10 was a factor of 2.7 ± 0.3. In the absence of drug we measured current enhancement caused by a change in external pH over the same range. In five cells the mean enhancement was a factor of 1.3 ± 0.1. This change, which is caused by the effects of protons on the calcium channel (see Prod'hom, et al., 1987; Krafte and Kass, 1988) is a significantly smaller effect on current amplitude than that measured in the presence of drug ($P < 0.005$) (see Discussion).

## DISCUSSION

The principal new result that we report is that extracellular pH modifies both the onset of and recovery from block of calcium channels by the DHP derivative amlodipine. These effects are not due to changes in the DHP receptor because the kinetics of nisoldipine block are unchanged over this pH range. The simplest interpretation of our results is that the degree of ionization of the amlodipine molecule can affect access to the DHP receptor and that the drug-receptor complex can be influenced by external protons. The implications of this interpretation are discussed below.

### *Influence of Charge of Drug on Channel Block: Implications for Location of DHP Receptor*

Under conditions that favor neutral drug molecules, we find that the onset of and recovery from block of amlodipine resembles that of other previously described neutral DHP derivatives. In extracellular solutions buffered to pH 7.4, over 94% of amlodipine molecules exist in ionized form, and the rate of block is slowed if protocols are used that minimize channel openings. This suggests that the receptor for amlodipine is not directly accessible from the extracellular aqueous phase.

The development of block seen in Fig. 6 is consistent with contributions from neutral and charged molecules which are both present at $pH_o$ 7.4 (Eq. 1). The single-pulse block primarily reflects the activity of neutral molecules that do not require channel openings to gain access to the receptor. Augmented block caused by the application of longer pulses is most likely due to additional contributions of ionized molecules which require that channels open before drug molecules can interact with DHP receptors. This is consistent with previous interpretations of the blocking activity of nicardipine (Sanguinetti and Kass, 1984) which also exists in neutral and charged forms at $pH_o$ 7.4.

Recovery from block is ten times slower in $pH_o$ 7.4 than in $pH_o$ 10, is not measurable in more acidic extracellular solutions, and is always incomplete if $pH_o$ is 7.4 or less. This result suggests that the ionized drug molecule cannot easily leave the vicinity of the DHP receptor after channels have been blocked. However, our experi-

ments also clearly demonstrate that the ionized drug/receptor complex is accessible to extracellular protons because channels recover rapidly from block when external pH is raised from 6.0 or 7.4 to 10.0.

It is important to recognize the influence of external hydrogen ions on the Ca channel itself and rule out this contribution from our observations. DHPs shift the calcium channel relationship between steady-state inactivation and voltage in the hyperpolarizing direction (see Sanguinetti and Kass, 1984). In the absence of drug, Krafte and Kass (1988) have shown that a change in $pH_o$ from 7.4 to 10.0 will cause a small (−5 mV) hyperpolarizing shift of voltage-dependent gating. This shift will be larger (−15 mV) with a change in $pH_o$ of 6.0 to 10.0. However, because external alkalinization shifts gating in the same direction along the voltage axis as DHP compounds, gating shifts induced in $pH_o$ 10 would be expected to cause additional block, not the relief of block we observed.

Protons can also directly block calcium channels (Prod'hom et al., 1987; Krafte and Kass, 1988). Raising external pH will thus relieve proton block of the channel and enhance current amplitude. However, in the present experiments the enhancement of available current by external alkalization in the presence of drug is much greater than the change in current measured upon a similar change in $pH_o$ in the absence of drug.

Our results are therefore consistent with relief of block in $pH_o$ 10.0 but not in $pH_o$ 7.4. We thus can conclude that protons must be able to access the drug/receptor complex possibly via a direct aqueous pathway between the drug-bound receptor and the extracellular solution. Furthermore, we find that $pH_o$-induced relief of block occurs under conditions in which channels are completely blocked in $pH_o$ 7.4 and at voltages that promote low probabilities of channel openings (−80 mV). Thus it appears that channels need not be opened for protons to gain access to the drug-receptor complex.

### *Similarities to Local Anesthetic Block of Sodium Channels: the Modulated Receptor Hypothesis*

Our results resemble the effects of $pH_o$ on sodium channel block by local anesthetics (Hille, 1977*b*; Schwarz et al., 1977; Grant et al., 1980). The work on sodium channels led to the hypothesis (Hille, 1977*a*; Hondeghem and Katzung, 1977) that the drug receptor for local anesthetic molecules is within the Na channel and that drug molecules come and go from it via the membrane phase (hydrophobic pathway) or via the inner mouth of the channel (hydrophilic pathway). The fact that external pH modified use-dependent block led Schwarz et al. (1977) to postulate that drug molecules bound to the receptor were free to acquire or give up protons via the external mouth of the channel. As a result, the site of the receptor was thought to be within the channel between the region associated with the selectivity filter and the intracellular mouth (see also Hille, 1984).

Electrical studies of calcium channels have provided some estimates of channel dimensions and mechanisms of ion permeation (Almers and McCleskey, 1984; Hess et al., 1986). This channel is thought to differ from the sodium channel in that selectivity occurs by affinity and not by molecular sieving. The estimate of the outer mouth of the calcium channel pore, ~6 Å, is close to the predicted dimensions of

DHP molecules (Rhodes et al., 1985). Thus, it is possible that ionized DHP molecules can pass through the outer mouth of the open channel and reach the DHP receptor.

One explanation for our findings, therefore, is that the DHP receptor is located within the calcium channel and that, as is the case for local anesthetics, charged and neutral DHP molecules reach the receptor via hydrophobic or hydrophilic pathways. In nerve sodium channels, Cahalan and Almers (1979) have shown that a drug-bound receptor located within a channel pore can inhibit current through that pore by interacting with gating instead of simply by obstruction. Thus the location of a DHP receptor within the calcium channel pore does not exclude the widely held view that these compounds regulate calcium channels via modification of gating.

### *An Alternate Interpretation: Membrane-bound Pathways for Both Neutral and Ionized Molecules*

Rhodes et al. (1985) and Chester et al. (1987) have modeled DHP binding via lipid or aqueous approaches and have measured diffusional dynamics of DHP probes in membrane preparations. These investigators conclude that neutral DHP molecules reach receptors via the membrane pathway. The very high partition coefficients for DHP derivatives partitioning into lipids from aqueous environments (Rhodes et al., 1985) suggests that even charged forms of these drugs may follow a membrane pathway. Electron density profiles of amlodipine indicate that this drug also partitions into cardiac sarcolemmal lipid bilayers (Mason et al., 1988). The location of the drug molecule is near the hydrocarbon core/water interface with the charged region of the molecule extending into polar headgroups of the membrane lipid bilayer. The position of amlodipine at pH 7.4 is slightly closer than uncharged DHPs to the polar headgroup. Channel conformational changes that accompany voltage-dependent gating transitions would then change access of the DHP receptor to membrane-bound drugs. Access of neutral and charged drug molecules would differ, and external hydrogen ions would certainly be free to titrate the ionized drug-bound receptor. We cannot exclude this possibility for drug access to the DHP receptor from our experiments.

One observation in its favor is that the voltage-dependent effects of amlodipine persist for long periods of time after drug has been washed from the extracellular solution. We have also found that the period of exposure of cells to a fixed concentration of amlodipine in the bulk solution affects the number of channels blocked by a given voltage protocol, but not the time course of recovery from block. This suggests that drug concentration in the membrane changes with prolonged exposure to extracellular drug molecules, and that the concentration of drug in the membrane determines drug-receptor interactions. In addition, Valdivia and Coronado (1988) have provided a pharmacological profile of skeletal muscle calcium channels incorporated into lipid bilayers and have suggested that the DHP receptor for these channels is buried in the lipid bilayer adjacent to the external end of the channel. Our results are consistent with this interpretation.

In our cells, we tried to determine whether access to the DHP receptor requires entry via the intracellular channel mouth by including amlodipine in patch pipettes

buffered to pH 7.4. In these experiments we tested for amlodipine effects by measuring appearance of voltage-dependent block after establishing whole-cell recording conditions. A similar approach by Heschlker et al. (1982) was useful in demonstrating that access to the receptor for D600 occurs from the intracellular face of the cell membrane. In seven experiments we found no evidence for drug-induced block when the drug was applied via the pipette despite waiting for periods in excess of 15 min and using drug concentrations of 10 $\mu$M. These experiments are suggestive, but not conclusive, that amlodipine molecules access the DHP receptor via the extracellular face of the membrane, which is consistent with the findings of Valdiva and Coronado (1988). However, experiments must be carried out to determine the distribution of drug within the cell as a function of time and distance from application via the pipette before conclusions can be drawn about the importance of intracellular application (Pusch and Neher, 1988).

*Relationship to Previous Studies of $I_{Ca}$ Inhibition*

This represents the first study in which the effects of extracellular pH were monitored on the blocking activity of one DHP compound. Uehara and Hume (1985) investigated the influence of external acidification on a diverse group of compounds that block calcium channels. They found that external acidification did not affect the kinetics of recovery from block by diltiazem, a non-DHP calcium channel blocker, and speculated that a proposed binding site responsible for use-dependent block by DHP compounds was proton inaccessible. Most of our experiments focus on external alkalinization. We clearly find that increasing external pH does not affect nisoldipine block of $I_{Ca}$ but has marked effects on amlodipine block of this current. We conclude from these results that modification of $pH_o$, over the range tested, does not alter the receptor site responsible for DHP block of the channel, but that the changes we observe in amlodipine block are due to alteration of the ratio of charged to neutral drug molecules. Because diltiazem binds to sites distinct from DHP receptors (Glossman et al., 1984), it is likely that the previous conclusions were based on drug binding to a different receptor than that studied in the present investigation.

*Conclusions*

Our experiments demonstrate that extracellular pH modifies block of $I_{Ca}$ by the DHP amlodipine in a manner that strikingly parallels the effects of $pH_o$ modification of sodium channel block by local anesthetics. We find that external protons can interact with the drug-bound DHP receptor and this interaction can occur when channels are blocked and closed. Our results imply that if the DHP receptor is located within the calcium channel itself, a pathway linking it to extracellular protons must exist even when the channel is closed. Alternatively, the DHP receptor may be located within the lipid bilayer but, if this is the case, then the charged group of the bound drug must still be accessible to extracellular protons, placing the DHP receptor near the external face of the sarcolemmal membrane.

This work was supported by United States Public Health Services grants HL-21922 (R. S. Kass) and HL-07678 (J. P. Arena).

We thank Donna Dimmano for her technical support in carrying out these experiments.

*Original version received 7 June 1988 and accepted version received 13 December 1988.*

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