Nitrendipine block of cardiac calcium channels: High-affinity binding to the inactivated state

(voltage clamp/Ca\(^{2+}\) antagonists/modulated receptor hypothesis)

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ABSTRACT Block of Ca\(^{2+}\) currents by the dihydropyridine drug nitrendipine was studied in single canine ventricular cells by using the whole-cell variant of the patch-clamp technique. When cells were held at depolarized membrane potentials at which Ca\(^{2+}\) currents were \(\approx 70\%\) inactivated, nitrendipine blocked Ca\(^{2+}\) currents very potently, with half-block by subnanomolar concentrations. The concentration dependence of block had the form expected for 1:1 binding, with an apparent dissociation constant (\(K_d\)) of 0.36 nM. In contrast, when cells were held at hyperpolarized potentials, nitrendipine blocked Ca\(^{2+}\) currents much less potently (\(K_d \approx 700\) nM). The results can be explained if nitrendipine binds very tightly to the inactivated state of the Ca\(^{2+}\) channel and only weakly to the normal resting state. The \(K_d\) estimated for binding to the inactivated state is very similar to the dissociation constants previously found for high-affinity \([\text{H}]\)nitrendipine binding to membrane fragments from heart, smooth muscle, brain, and other tissues; moreover, the concentration-dependent kinetics of binding to the inactivated state are similar to those reported for \([\text{H}]\)nitrendipine binding to membranes. These results make it seem very likely that the high-affinity \([\text{H}]\)nitrendipine binding site is an inactivated state of the Ca\(^{2+}\) channel.

Ca\(^{2+}\) channels are found in many kinds of cells and are important for electrical excitability, excitation–contraction coupling, excitation–secretion coupling, and other cellular functions (1, 2). The development of drugs that block Ca\(^{2+}\) channels has provided a valuable route for studying channel function, and there has been rapidly increasing clinical use of Ca\(^{2+}\) channel blockers in combating hypertension, angina, and arrhythmias (3). Recently, there has been particular interest in the family of dihydropyridine drugs related to nifedipine, especially since the discovery that \([\text{H}]\)nitrendipine and other radioactively labeled derivatives bind with very high affinity (\(K_d = 0.1-1\) nM) to sites in brain, heart, smooth muscle, and other tissues (4–7). Based on the Ca\(^{2+}\) channel-blocking activity of the drugs, it is widely hoped that the binding site now being purified in several laboratories with the aid of radioactive labeling (8, 9) will be a Ca\(^{2+}\) channel protein.

However, the relationship between dihydropyridine binding and block of Ca\(^{2+}\) channels has been unclear. In tissues like heart and brain, there is no known effect of low concentrations of dihydropyridines on physiological function despite the presence of high-affinity binding sites at a fairly high density (10, 11). Also, all voltage clamp studies so far have found that the dihydropyridine concentrations needed to block Ca\(^{2+}\) currents are 100–1000 times higher than would be expected from the binding data (12, 13). This discrepancy has raised the possibility that the high-affinity binding site is not related to block of Ca\(^{2+}\) channels.

Studying Ca\(^{2+}\) channels in voltage-clamped dog ventricular cells, I have found that Ca\(^{2+}\) current can be blocked by nanomolar concentrations of nitrendipine if the cells are held at membrane potentials at which most channels are inactivated. Interpreted by a modulated receptor hypothesis like that devised to account for voltage- and frequency-dependent features of local anesthetic block of Na\(^+\) channels (14, 15), the results suggest that nitrendipine binds to the inactivated state of the Ca\(^{2+}\) channel with a \(K_d\) of 0.25 nM. Just as for \([\text{H}]\)nitrendipine binding to the high-affinity site, the kinetics of Ca\(^{2+}\) current block by low nitrendipine concentrations are found to be slow, requiring many minutes to reach equilibrium. In addition to clarifying the relationship between high-affinity binding and channel blocking activity, the results may shed light on the tissue specificity with which dihydropyridines affect physiological function.

METHODS

Isolation of Cells. Beating hearts were removed from mongrel dogs of either sex and placed into ice-cold modified Tyrode’s solution containing (in mM) 150 NaCl, 4 KCl, 2 CaCl\(_2\), 2 MgCl\(_2\), 10 glucose, 10 Hepes (pH 7.3). A piece of muscle about 1 cm square was removed from near the center of the right ventricle, placed in 0 Ca Tyrode’s solution (same as above but with CaCl\(_2\) omitted), and minced with scissors into 1-mm pieces, which were then transferred into 10-ml beakers containing the enzyme solution [modified from Bus-tamente et al. (16)] of 0.05 mg of protease (Sigma type VII) per ml and 0.6 mg of collagenase (Sigma type 1) per ml in 0 Ca Tyrode’s solution. The pieces were stirred at 37°C for 20 min, trituated about 20 times with a broken-off Pasteur pipette, incubated another 20 min, trituated again, and then filtered through 200-μm nylon mesh. The filtrate was spun at \(\approx 1000\) rpm for 3 min and the pellet was resuspended in 0.5 mM Ca Tyrode’s solution. Cells were kept at room temperature and used within 24 hr; only relaxed, striated, rod-shaped cells were used.

Current Recording. Whole-cell voltage clamp currents were recorded following the method of Hamill et al. (17). Cells were placed in 2 mM Ca Tyrode’s solution and approached with fire-polished electrodes (2–4 MΩ) containing (in mM) 120 CsCl, 10 Cs\(_2\)EGTA, 5 MgCl\(_2\), 10 Hepes (pH 7.4). After formation of a gigaseal and rupture of the membrane, the currents were allowed to stabilize (2–10 min) and the external solution was then changed to the recording solution containing (in mM) 2–20 CaCl\(_2\) or BaCl\(_2\), 135 tetraethylammonium chloride, 2 MgCl\(_2\), 10 glucose, 10 Hepes (pH 7.4). These ionic conditions allowed optimal recording of Ca\(^{2+}\) currents: Na\(^+\) current was completely eliminated, K\(^+\)–channel currents were completely or nearly completely suppressed (see Fig. 1A Inset), and the input resistance of the cells was usually 1–5 GΩ. Bathing solution flowed through the chamber (capacity, \(\approx 1\) ml) at a constant rate of 5–15 ml/min throughout the experiment. Depolarizing pulses were given at intervals of 9–30 s to avoid possible use-dependent effects. Membrane currents were filtered at 1 KHz, digitized at intervals of 100–300 μs, and stored and analyzed on a computer.

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laboratory computer. For measurement of peak Ca$^{2+}$ current, leak and capacity currents were subtracted by using current from hyperpolarizing pulses, and peak inward current was read as the average of ~10 points around the peak. Experiments were done at 22°C.

**Drug Application.** Nitrendipine (kindly supplied by Alexander Scriabine, Miles Laboratories, New Haven, CT) was dissolved as a 2 or 10 mM solution in ethanol, prepared daily and protected from light. Aliquots were serially diluted in the external aqueous solution to the final concentration. In control experiments at depolarized holding potentials, concentrations of ethanol 100 times higher than those in the largest nitrendipine solutions were found to have no effect on Ca$^{2+}$ current; at hyperpolarized potentials, ethanol concentrations equivalent to those in the 10 μM nitrendipine solution had no effect. Separate chambers, solution reservoirs, and tubing were used for experiments with low (0.3–10 nM) and high (0.1–30 μM) nitrendipine concentrations and were rinsed repeatedly with ethanol between nitrendipine applications.

**RESULTS**

**Block at Depolarized Holding Potentials.** Fig. 1 illustrates potent nitrendipine block of Ca$^{2+}$ currents in cells held at depolarized membrane potentials. The experiment in Fig. 1A was done with a cell held at −10 mV, at which Ca$^{2+}$ current was about 70% inactivated compared with the current elicited from a holding potential of −80 mV. As in other kinds of cells that are internally dialyzed (18–20), the size of the Ca$^{2+}$ current showed a slow decline even in the absence of drug. Fortunately, this decline was slow enough that drug block of current could be clearly distinguished from continuing baseline rundown. The amount of block (to 23% of control in Fig. 1A) could be estimated by recording control currents for a long period (26 min in Fig. 1A, of which the final 11 min are shown) before drug was applied and assuming that normal rundown continued to occur during drug block. In two other applications of 1 nM nitrendipine under similar conditions, block to 19% and 25% of control was observed.

The experiment in Fig. 1A was typical of the other experiments with 1 nM nitrendipine in showing a relatively slow block of current (half-time, 2½–4 min) after nitrendipine was applied; this was much longer than the time needed for exchange of solution in the chamber (half-time, 15–45 s).

Larger drug concentrations blocked more completely and more quickly. Fig. 1B shows that 10 nM nitrendipine blocked Ca$^{2+}$ current completely, with a half-time of about 1 min. When cells could be voltage-clamped for long enough, washing the cell with drug-free solution after nitrendipine block almost always resulted in some recovery of current size, as in Fig. 1B. The recovery of current on rinsing suggests that nitrendipine acts by a reversible blocking action on Ca$^{2+}$ channels, rather than (for example) simply accelerating the normal rundown process. The time course of recovery was always slow, as in Fig. 1B, with current continuing to increase for 10–20 min; because of the slowness of recovery and the small underlying continual rundown of Ca$^{2+}$ current, recovery was never more than partial.

The results in Fig. 1A and B were typical of >40 applications of nanomolar concentrations of nitrendipine to dog heart cells held at depolarized membrane potentials. Fig. 1C shows the concentration dependence of nitrendipine block in 12 cells bathed in a solution containing 20 mM Ca$^{2+}$. Data were restricted to cells for which long baseline series of control currents were recorded (>7 min) and in which the rate of rundown was slow enough (<3.5%/min) to allow optimal separation of nitrendipine block from normal rundown. The holding potential varied from −10 to −20 mV and was adjusted in each cell so that steady-state inactivation was >2/3 complete. Under these conditions, the dose dependence of nitrendipine block can be fit very well by a curve having the form expected from 1:1 binding, with an apparent $K_d$ of 0.36 nM.

Fig. 1D shows the concentration dependence of the rate of block by nitrendipine. The rate of block increased markedly as the nitrendipine concentration increased. The data are fit well by a curve drawn according to a simple model of nitrendipine binding and block (see Discussion).

In addition to the discrepancy previously reported between nitrendipine concentrations needed for binding or blocking, there has been an apparent difference in modulation of the two processes by divalent ions. Lee and Tsien (13) found that the blocking potency of large concentrations of nitrendipine could be changed considerably by varying external Ca$^{2+}$ or by substituting Ba$^{2+}$ for Ca$^{2+}$; in contrast, binding studies have shown very small effects of changing Ca$^{2+}$ or Ba$^{2+}$ concentrations from 1 to 100 mM (21, 22). In experiments like those in Fig. 1A, nitrendipine block was also
not much affected by the divalent ion species or concentration. Fig. 2 summarizes experiments comparing block by 1 nM nitrendipine in bathing solutions containing 2 mM Ca\(^{2+}\), 20 mM Ca\(^{2+}\), and 20 mM Ba\(^{2+}\). In all cases, nitrendipine reduced currents to 20–30% of control. Nitrendipine was equally potent in other experiments at depolarized holding potentials using bathing solutions of isotonic (110 mM) BaCl\(_2\), suggesting that the tetraethylammonium chloride present in most experiments had little effect on block. Experiments on cells isolated from the right atrium or from the sino-atrial node showed equally potent Ca\(^{2+}\) current block.

**Block at More Negative Holding Potentials.** When cells were held at negative membrane potentials at which channels were not inactivated, nitrendipine concentrations >1000 times higher were required for equivalent Ca\(^{2+}\) channel block. Fig. 3A shows a typical example in which Ca\(^{2+}\) currents were elicited from a holding potential of −80 mV; application of 1 μM nitrendipine resulted in prompt reduction of current to 50% of control, and currents recovered substantially within a few minutes of rinsing with drug-free solution. In other cells, hyperpolarizing the membrane beyond −80 mV was found to have no effect on current size either in the absence or presence of 1 μM nitrendipine, suggesting that the effect of holding potential on nitrendipine potency is saturated at −80 mV. In contrast to the slow development of block seen with nanomolar concentrations at depolarized potentials, block by micromolar concentrations at negative holding potentials developed quickly (half-time 15–45 s), with the speed probably limited by the rate of solution exchange. Recovery was also rapid (half-time, 45–90 s). If block was induced by 1 mM nitrendipine at more depolarized holding potentials, changing the holding potential to −80 mV resulted in prompt removal of block.

Fig. 3B shows the concentration dependence of nitrendipine block of Ca\(^{2+}\) currents elicited from −80 mV in 20 mM Ca\(^{2+}\) bathing solution. Block increased over the range of 0.1–3 μM nitrendipine in a manner fairly well described by 1:1 drug-to-receptor binding with a K\(_d\) of 0.73 μM. However, there was considerably less block at higher concentrations than expected from this mechanism. Even solutions of nominally 30 μM nitrendipine left >10% of the inward current unblocked. The residual inward current probably did not arise from Ca\(^{2+}\) flowing through Na\(^{+}\) channels since it was not blocked by 12 μM tetrodotoxin. It was not an artifact of capacity or leak current subtraction, since it was eliminated by substituting Co\(^{2+}\) for Ca\(^{2+}\).

As with block at depolarized potentials, block by large concentrations at negative holding potentials was not much affected by changing Ca\(^{2+}\) concentrations or by using Ba\(^{2+}\) as the current-carrying ion. As shown in Fig. 4, 1 μM nitrendipine blocked by the same amount in 2 mM Ca\(^{2+}\), 20 mM Ca\(^{2+}\), and 20 mM Ba\(^{2+}\).

**DISCUSSION**

The principal finding of this study is that nitrendipine blocks cardiac calcium currents very potently at holding potentials at which most channels are inactivated. At more negative holding potentials, block is less potent by a factor of >1000.

These results can be understood by postulating that nitrendipine binds tightly to the inactivated state of the Ca\(^{2+}\) channel and much more weakly to the resting state. This interpretation is a version of the modulated receptor hypothesis, successfully used to account for voltage-dependent and frequency-dependent aspects of local anesthetic block of Na\(^{+}\) channels (14, 15, 23, 24).

Fig. 5 shows a version of the modulated receptor model that can account quantitatively for the results described in this paper. With no drug, a Ca\(^{2+}\) channel can be in either the resting state (R), in which it is closed but available to be opened by a depolarization, or in the inactivated state (I), in which it is closed and not available to be opened. Experimentally, the number of channels in state R is assayed by the Ca\(^{2+}\) current elicited by a large depolarization. The distribution of channels between states R and I depends on the steady membrane potential, with more channels in the inactivated state at depolarized holding potentials.
FIG. 4. Block by 1 μM nitrendipine. Bars show mean ± SEM for determinations in 3 (2 mM Ca, 20 mM Ba) or 8 (20 mM Ca) cells. Data for 20 mM Ca as in Fig. 3. In 2 mM Ca or 20 mM Ba, block was determined by using the test pulse to +10 mV (peak of I-V curve) from −80 mV.

At very negative holding potentials, all channels will be in state R; nitrendipine block will simply follow the 1:1 binding of drug to receptor with dissociation constant $K_R$. At depolarized holding potentials at which some channels are in state I, nitrendipine block of currents is more complicated. For example, if 1 nM nitrendipine is applied, there will be essentially no binding to channels in the resting state but considerable binding to inactivated channels, with a dissociation constant $K_I$. Binding to inactivated channels is electrically silent in itself, since the channels are already nonconducting and not available for opening, but because of the equilibrium between resting and inactivated channels, decreasing the fraction of channels in state I will cause a proportionate decrease in the fraction in state R and thus produce a smaller peak Ca$^{2+}$ current elicited by depolarization. Using the scheme in Fig. 5 to solve for the fraction of channels in R as a function of nitrendipine concentration, one finds that the concentration dependence of Ca$^{2+}$ current block is expected to follow the form expected from simple 1:1 binding, but with an apparent dissociation constant that is determined by a mix of the actual binding reactions:

$$K_{app} = \frac{1}{(h/K_R) + (1-h)/K_I},$$

in which $h$ is the fraction of channels in state R in the absence of drug. The data in Fig. 1C are clearly consistent with the predicted form. With $K_R >> K_I$, $K_I = (1-h)K_{app}$. In the experiments summarized in Fig. 1C, $h$ was about 0.3, and thus the observed apparent dissociation constant of 0.36 nM would correspond to an actual dissociation constant of 0.25 nM for binding to the inactivated state.

The model in Fig. 5 is also consistent with the concentration dependence of channels between resting and inactivated states is rapid compared with nitrendipine binding (consistent with the relaxing of milliseconds and seconds that predominate in the absence of drug), nitrendipine block will develop with a time constant equal to $t_1=(1-h)N-k_I$, in which N is the nitrendipine concentration. Since the ratio $k_{-1}/k_I = (K_R)$ is determined by the fit to the equilibrium dose–response curve, the expression for the expected time constant can be fit to the experimental data with $k_{-1}$ as a free parameter; as Fig. 1D shows, the model accurately predicts the form of the experimental data, and a value of 0.0012 s$^{-1}$ is obtained for $k_{-1}$.

Lee and Tsien (13) found half-block of Ca$^{2+}$ currents by 154 nM nitrendipine. Since they used a holding potential of −40 mV, at which channels would be mostly, but not completely, in the resting state, their value fits well with the model in Fig. 5 in being much closer to the $K_d$ for resting channels than inactivated channels; there would be quantitative agreement if 99.9% of the channels were in the resting state in their experiments. At such intermediate potentials, the apparent dissociation constant would be quite sensitive to very small changes in $h$ and might show considerable cell to cell variability.

As shown in Figs. 2 and 4, neither changing the Ca$^{2+}$ concentration nor substituting Ba$^{2+}$ for Ca$^{2+}$ had much effect on nitrendipine’s potency either at depolarized or hyperpolarized potentials, suggesting that binding to both resting and inactivated states is insensitive to external divalent. These results are in apparent contrast to those of Lee and Tsien, who found that block was much stronger with Ba$^{2+}$ than Ba$^{2+}$ and was antagonized by increasing Ca$^{2+}$ concentration. A likely explanation for the Ca$^{2+}$ antagonism in their experiments is that increasing external Ca$^{2+}$ will, by changing the external surface charge of the membrane, lead to an effective hyperpolarization and a shift of the apparent dissociation constant closer to the true dissociation constant for the resting state, in the absence of any true change in the affinity of nitrendipine for either resting or inactivated channels. It is less obvious why substituting Ba$^{2+}$ for Ca$^{2+}$ at an intermediate potential would lead to apparent antagonism of block, but the data in Figs. 2 and 4 suggest that this effect is probably not an actual change in the nitrendipine binding affinity for either state of the channel.

An initial study of Ca$^{2+}$ channel block by large nisoldipine concentrations reported little or no voltage dependence (12). However, the results reported here seem consistent with recent abstracts by Sanguinetti and Kass (25) and Uehara and Hume (26) reporting voltage-dependent block by nisoldipine and nifedipine. The model in Fig. 5 predicts large hyperpolarizing shifts in the availability curve for Ca$^{2+}$ current, as were apparently seen in these studies. In fact, with large concentration of drug, the midpoint of the curve would shift by $k_{-1}(K_R/K_d)$, in which $k$ is the slope factor of the curve; with $k = 10$ mV, as in dog heart cells (unpublished observations), a shift of up to 70 mV would be predicted.

FIG. 5. Modulated receptor model for nitrendipine block of Ca$^{2+}$ channels. One-to-one binding of drug is assumed, with different dissociation constants for resting and inactivated channels. Binding to open channels is omitted; it is probably minimal below 10 nM, since the time course of inactivation is unchanged (e.g., Fig. 1A Insert). At very high concentrations, open channel block may occur (see ref. 13) and would lead to an underestimate of $K_R$. An alternative to this model would be to suppose that drug binds only to inactivated channels, but that even at very negative potentials, a tiny fraction of channels remains inactivated. If this fraction were 0.0025%, this alternative would fit the equilibrium dose–response data. However, it would predict that half-block by 1 μM drug at −80 mV would be equally slow as half-block by 0.3 nM drug at depolarized holding potentials, in contrast to the observations (Figs. 1D and 3A).
However, shifts at nanomolar concentrations would be difficult to detect, since the equilibration of drug block takes minutes, compared to the preps of milliseconds or seconds used in conventional protocols. The assumption that nitrrendipine binds to a single inactivated state may be oversimplified. As in other cells, inactivation of Ca currents in heart cells seems to be promoted by Ca entry itself as well as by a voltage-dependent mechanism (see ref. 2 for a review). Ca-dependent inactivation is unlikely to have contributed to the results reported here, since cells contained EGTA and results were identical when Ba was used as the current carrier (Fig. 2). It would be interesting to examine nitrrendipine block under conditions in which Ca-dependent inactivation is more important.

The $K_d$ of 0.25 nM for nitrrendipine binding to inactivated channels estimated from Figs. 1 and 5 is very close to the $K_{ds}$ of 0.1–0.4 nM that have most often been obtained for high-affinity [3H]nitrrendipine binding to cardiac muscle from dog and other species (4–7, 27, 28). Also, the kinetics of block (Fig. 1) are very similar to the kinetics of [3H]nitrrendipine binding obtained at similar temperatures (21, 29); for example, the estimate of $10^{-3} \text{ s}^{-1}$ for the rate constant $k_1$ is identical to that by Bolger et al. (29). Further, in the block and experiments described here and in previous binding studies, both the kinetics and steady-state characteristics of block or binding can be fit accurately by assuming 1:1 binding of drug to the high-affinity receptor. Finally, the lack of change in blocking potency when Ca$^{2+}$ is varied in the millimolar range is consistent with the properties of binding to the high-affinity site (21, 22). Taken together, all of these observations provide strong support for the idea that the high-affinity dihydropyridine binding site is associated with the inactivated state of the Ca$^{2+}$ channel, a possibility raised previously by Lee and Tsien (13). This idea offers a satisfying explanation for the previous discrepancy between blocking and binding. Previous determinations of blocking potency were carried out from holding potentials at which almost all channels would be in the low-affinity resting state, while binding studies are usually performed by using membrane fragments, in which the Ca$^{2+}$ channel proteins presumably sense a membrane potential near 0 mV and all channels are inactivated. One binding study employed enzymatically isolated intact cells (30), but it is possible that the high-affinity binding sites were all associated with the 10% of the cells that are damaged and depolarized in even the best single cell preparations. Alternatively, it is possible that the high-affinity site is present in resting channels but that occupancy does not block the channel. In its simplest form, though, the model in Fig. 5 predicts little or no high-affinity binding to well-polarized tissue, and drastically depolarizing intact tissue (as with large K$^+$ concentrations) or purposefully disrupting intact cells should result in the appearance of high-affinity binding sites.

The results offer a simple explanation of why low concentrations of dihydropyridines affect the contractility of smooth muscle but, so far as is known, have no effect on normal heart or brain function (10, 11, 31). In the normal heart, it is very unlikely that any region reaches a depolarized membrane potential for long enough to permit significant dihydropyridine binding; though Ca$^{2+}$ channels certainly become inactivated during the action potential plateau, the time a channel spends in the inactivated state ($<1 \text{ s}$) is much less than the minutes it takes nitrrendipine to bind to inactivated channels (Fig. 1). The situation in neurons may well be similar. In contrast, dihydropyridine effects on smooth muscle contractility are usually evaluated in vitro by using K$^+$ depolarizations that are maintained for minutes; the slow binding to the inactivated state also offers a ready explanation for why tonic contractures are more potent than phasic contractures in these experiments (32, 33). It is possible that similarly long depolarizations occur in vivo, perhaps due to tonic sympathetic activity. Thus, the blocking characteristics of low concentrations of drug may account for the tissue specificity of drug action without having to postulate any fundamental difference in the way that Ca$^{2+}$ channels in the different tissues interact with drug molecules. Performing similar experiments on Ca$^{2+}$ channels in smooth muscle and neuronal cells will permit a test of this idea.

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