Effect of Membrane Depolarization on Binding of [³H]Nitrendipine to Rat Cardiac Myocytes

Frank J. Green, Barbara B. Farmer, Gail L. Wiseman, Mimi J.L. Jose, and August M. Watanabe
From the Departments of Medicine and Pharmacology, and the Krannert Institute of Cardiology, Indiana University School of Medicine, Indianapolis, Indiana

SUMMARY. Binding of the dihydropyridine calcium antagonist [³H]nitrendipine was studied in cardiac myocytes incubated in normal and high potassium buffer so that we might examine the voltage dependence of dihydropyridine binding. Hearts were obtained from adult male Wistar rats, and isolated calcium tolerant myocytes were dissociated by enzymatic dispersion. Cells in 5.6 mM extracellular potassium showed specific binding of [³H]nitrendipine with KD 587 ± 50 (mean ± se) pM and maximum receptor density, Bmax, 10.8 ± 1.3 fmol/mg wet weight. Cells depolarized in 50 mM potassium showed no change in KD, 661 ± 77 pM, but approximate doubling of Bmax, 25.6 ± 3.7 fmol/mg wet weight. Binding equilibrium was reached within 5 minutes at 37°C, and the KD determined by kinetic analysis was in good agreement with KD determined by saturation experiments. Unlabeled nitrendipine and nifedipine completely inhibited [³H]nitrendipine binding with slope factors less than one, whereas verapamil and diltiazem only partially inhibited binding with slope factors substantially less than one. As a function of increasing extracellular potassium concentration from 2.4-54.1 mM, the number of nitrendipine-binding sites increased gradually 115%. Aconitine also produced a 58% increase in binding sites over a concentration range of 1-30 µg/ml. The potassium-induced changes in number of binding sites occurred rapidly and were rapidly reversible with changes in extracellular potassium concentration. There was no change in the number of [³H]nitrendipine-binding sites as a function of potassium or aconitine concentration in dead or digitonin-treated myocytes. We conclude that nitrendipine receptor density in viable myocytes is voltage dependent, but we detect no change in KD as a function of voltage. (Circ Res 56: 576-585, 1985)

THE calcium channel in cardiac tissue has been the subject of intense scrutiny during the past decade (Reuter, 1983; Hagiwara and Byerly, 1981; Tsien, 1983). Recent advances in electrophysiological techniques, including voltage-clamp studies and gigaseal recordings of membrane patches in isolated cardiac cells, have increased our understanding of the kinetics of the calcium channel and its response to depolarization, adrenergic agonists, cyclic adenosine monophosphate analogs, cAMP-dependent protein kinase, and calcium antagonists (Reuter, 1983; Lee and Tsien, 1983; Fenwick et al., 1982; Osterrieder et al., 1982). Several investigators have observed that the calcium channel exists in one open and two closed states. When it is open, it admits a current of uniform amplitude, which suggests that there is no intermediate conductance through the channel. Depolarization of the membrane increases the probability of channel opening (Fenwick et al., 1982; Reuter, 1982, 1983). Recently, calcium channel behavior has been characterized as having three gating modes, comprised of periods of no opening of the channel, brief openings, or prolonged openings and only brief closings (Hess et al., 1984).

The interaction of calcium channel antagonists with the channel recently has been further delineated. Verapamil, diltiazem, and nitrendipine block both inward and outward current flow through the channel, and this is interpreted to be consistent with channel blockade. The fact that inward current can be restored by increasing extracellular calcium concentrations suggests that unblocked calcium channels are available for recruitment. The half-time of the decay of inward current is more rapid in the presence of calcium antagonists, and this has been interpreted to be consistent with binding of the drugs to open channels (Lee and Tsien, 1983). Recently, it has been noted that the effects of three dihydropyridine calcium channel antagonists, nisoldipine, nitrendipine, and nicardipine, on the calcium channel current, are augmented at less negative resting membrane potentials. This evidence of voltage dependence has been interpreted in terms of the modulated receptor hypothesis (Hondeghem and Katzung, 1984) and suggests that these drugs bind to inactivated channels as well (Sanguinetti and Kass, 1984). The concept that dihydropyridines act by sterically plugging the channel is being modified in favor of a model in which binding of the dihydropyridine stabilizes the channel in a mode in which no openings occur (Hess et al., 1984; Janis et al., 1984).

Particular interest in the dihydropyridine class of calcium antagonists has been sparked by the obser-
vations that \[^{3}H\]nitrendipine and \[^{3}H\]nimodipine bind to high-affinity receptors in membranes of a variety of tissues, including brain, myocardium, ileal and vascular smooth muscle, and skeletal muscle (Belleman et al., 1981; Gould et al., 1982; Glossman et al., 1982; Murphy and Snyder, 1982; Bolger et al., 1982; Ehler et al., 1982a, 1982b; Yamamura et al., 1982; Williams and Tremble, 1982; DePover et al., 1982, 1983a, 1983b; Fairhurst et al., 1983; Fosset et al., 1983; Sarmiento et al., 1983). In most of these studies, the measured equilibrium dissociation constant, \(K_D\), has ranged between 0.11 and 0.4 nM. A second site of lower affinity with \(K_D\) of 67 nM has been reported (Belleman, 1981). In addition, specific binding of \[^{3}H\]nitrendipine (Marsh et al., 1983) and of \[^{3}H\]nimodipine (DePover et al., 1983a) has been described in intact cells. The interaction of dihydropyridines with these sites is stereospecific, and correlation of pharmacological potency with order of binding affinity has been established in ileal smooth muscle (Bolger et al., 1982). In myocardium, however, it is well recognized that the \(K_D\) of the dihydropyridine receptor for \[^{3}H\]nitrendipine is two to three orders of magnitude lower than the concentration of nitrendipine which produces half-maximal pharmacological effects (Millard et al., 1983; Lee and Tsien, 1983). Because the effect of nitrendipine on calcium channel current is voltage dependent, a model has been proposed which postulates an increase in the affinity of the calcium channel for nitrendipine at less negative membrane potentials (Sanguinetti and Kass, 1984). Alternatively, the pharmacological IC$_{50}$ of nitrendipine for depression of contractility has been correlated with a second class of binding sites with lower affinity in cultured chick myocytes (Marsh et al., 1983) and in canine cardiac sarcolemma (Vaghy et al., 1984).

Competition between labeled dihydropyridines and unlabeled calcium channel antagonists of other chemical classes have disclosed apparent different sites of action of these drugs in myocardial membranes. Verapamil and D600 only partially inhibit \[^{3}H\]nitrendipine binding, increase the half-time of association and dissociation, and cause a maximum 4-fold increase in the equilibrium dissociation constant, \(K_D\), without changing the maximum number of receptor sites in rat cerebral cortex and myocardial homogenates (Ehler et al., 1982b). This interaction is postulated to be consistent with negative heterotropic cooperativity. In contrast, diltiazem has been observed to inhibit \[^{3}H\]nitrendipine binding partially at 0°C, whereas at 25°C and 37°C, partial or no inhibition, or actual enhancement, of binding occurs (Murphy and Snyder, 1982; Ehler et al., 1982a, 1982b; Yamamura et al., 1982; Ferry and Glossman, 1982; DePover et al., 1982). In brain membranes, diltiazem decreases the rate of dissociation of \[^{3}H\]nitrendipine from its receptor (Ferry and Glossman, 1982; Yamamura et al., 1982). In myocardial membranes, diltiazem has been reported to increase \[^{3}H\]nitrendipine- and \[^{3}H\]nimodipine-binding sites without changing receptor affinity (Millard et al., 1982; DePover et al., 1982, 1983b). In rabbit ventricular membranes, a diltiazem-induced increase in receptor affinity has been noted (Janis et al., 1984). In addition, diltiazem, in small concentrations that depress myocardial contractility only slightly, has been shown to potentiate the negative inotropic effects of nimodipine in isolated rat hearts (DePover et al., 1983b). These observations are consistent with allosteric modulation of the dihydropyridine-binding site by verapamil, but the mechanism of interaction with diltiazem appears to be more complex.

We studied \[^{3}H\]nitrendipine binding to intact polarized and depolarized ventricular myocytes in order to relate specific binding to changes in membrane voltage. We were interested in the following questions. What is the relationship between binding of \[^{3}H\]nitrendipine to high-affinity sites and membrane potential of the cell? If changes in \[^{3}H\]nitrendipine binding occur in response to alterations in membrane potential, are they reversible? Do calcium channel antagonists compete with \[^{3}H\]nitrendipine with different potency in polarized and depolarized cells? Does diltiazem increase the number of \[^{3}H\]nitrendipine-binding sites in intact cells?

**Methods**

**Myocyte Preparation**

Adult male Wistar rats weighing 250–300 g were injected with 500 U sodium heparin, intraperitoneally, and were killed 30 minutes later. Following a blow to the cranium, cervical dislocation was performed, and the hearts were rapidly excised and placed in ice-cold physiological saline solution. The aorta was transected adjacent to the innominate artery, cannulated, and subjected to retrograde perfusion at a constant pressure of 60 cm water with Krebs-Henseleit solution containing compounds of the following millimolar concentrations: NaCl, 118; KCl, 4.74; KH$_2$PO$_4$, 0.93; MgSO$_4$, 1.2; NaHCO$_3$, 25; glucose, 10; and CaCl$_2$, 2.5. The buffer was equilibrated with 95% O$_2$/5% CO$_2$ for 30 minutes. Calcium-tolerant myocytes were isolated enzymatically by a previously described method (Farmer et al., 1983). Briefly, following 20 minutes of perfusion at constant pressure with Krebs-Henseleit buffer containing 2.5 mM calcium, the hearts were subjected to perfusion with buffer devoid of calcium at a constant flow rate of 7.5–8 ml/min for 3.5–4.5 minutes. Next, Krebs-Henseleit buffer containing 0.1% collagenase, 0.1% bovine serum albumin, and 50 μM calcium was introduced via stopcock, and perfusion continued 5 minutes. The ventricles were then cut down, two vertical slabs were made toward but not including the apex, and the tissue then was immersed in Krebs-Henseleit buffer containing 0.1% collagenase (Worthington type I or Sigma type I), 2% bovine serum albumin, and 50 μM calcium and was shaken gently (Labline Orbit Shaker) for four 5-minute periods, each followed by sieving to harvest eluted cells. The cells were suspended and centrifuged twice in Krebs-Henseleit medium containing 50 μM calcium and 2% bovine serum albumin before final suspension in Krebs-Henseleit medium containing 1.8 mM calcium. Dead myocytes were prepared by chilling myocytes at 4°C for...
24–48 hours. In selected experiments, myocytes were made permeable by the addition of digitonin, 5 μg/ml.

Membrane Vesicles

Canine cardiac membrane vesicles were prepared as previously described by Jones et al. (1979).

Buffer

The myocytes were incubated in buffer with various concentrations of extracellular potassium, [K+]o. For [K+]o of 5.6, buffer constituents were the same as in the Krebs-Henseleit buffer used for final suspension. For selected experiments, buffer containing [K+]o of 2.5, 50, and 80 mM was created by adjusting KCl and increasing or reducing NaCl appropriately to maintain constant osmolarity. In addition, we also conducted experiments in which N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid (HEPES), 10 mM, or morpholinopropane sulfonic acid (MOPS), 20 mM, was substituted for NaHCO₃, with added NaCl to maintain constant osmolarity. Bicarbonate buffer was gassed with 95% O₂, 5% CO₂ to maintain pH 7.4, while Good buffers were gassed with 100% O₂ and pH was adjusted to 7.4 with 6 mM NaOH.

Equilibrium-Binding Studies

Saturation of [3H]nitrendipine [5 methyl-3H]-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl)-3, 5-pyridine-dicarboxylic acid ethyl methyl ester]-binding sites in myocytes was examined at 37°C after 30 minutes of incubation. All binding studies were performed in the dark. Myocytes from one preparation were divided and suspended in each buffer at a concentration of 2–5 mg wet weight per ml. Binding reactions were terminated by adding various volumes of cold buffer that contained the identical [K+]o and were maintained at a temperature of 4°C. Cold buffer was also used for washing filtered samples. Three experimental designs were used for equilibrium studies. Method 1: identical 2- to 3-ml volumes of cells were incubated with [3H]nitrendipine, 50–1200 pM, in the absence or presence of unlabeled nitrendipine. Binding studies were performed at various volumes of cold buffer that contained the identical [K+]o, and were maintained at a temperature of 4°C. Cold buffer was also used for washing filtered samples. Three experimental designs were used for equilibrium studies. Method 1: identical 2- to 3-ml volumes of cells were incubated with [3H]nitrendipine, 50–1200 pM, in the absence or presence of unlabeled nitrendipine, 1 μM, and 200-μl aliquots were then sampled in triplicate for measurement of total and nonspecific binding, respectively. Method 2: identical 3-ml volumes of cells were incubated with [3H]nitrendipine. After 30 minutes, triplicate and duplicate 200-μl aliquots were taken for measurement of total binding and total counts, respectively. Unlabeled nitrendipine, 40 μM, was added in a volume of 50 μl to yield a final concentration of 1 μM. The suspension was incubated for 30 more minutes, and triplicate 200-μl aliquots were taken for measurement of nonspecific binding. For both methods 1 and 2, each sample aliquot was immersed for 15 minutes in a 5-ml volume of buffer. The 5-ml volumes were then filtered over Whatman GF/C filters and washed three times with 5 ml of buffer. Both methods gave comparable values for nonspecific binding. Method 3: triplicate 400-μl volumes of cells were incubated with ascending concentrations of [3H]nitrendipine in a final volume of 500 μl in the presence and absence of unlabeled nitrendipine, 1 μM. After 30 minutes of incubation at 37°C, the reaction was terminated by the addition of 10 ml of buffer. After 15 minutes, the 10-ml volume was filtered over Whatman GF/C glass fiber filters and washed three times with 5 ml of buffer.

We determined that the 15-minute immersion in cold buffer prior to filtration caused a net decrease in total and nonspecific binding without changing specific binding. For instance, at equilibrium in 400 pM [3H]nitrendipine, 0.5 mg wet weight of cells filtered immediately after immersion yielded total binding, 1716, nonspecific binding, 1066, and specific binding, 650 counts/min, whereas 15 minutes of immersion before filtering gave 1086, 499, and 587 counts/min, respectively. Thus, the percent specific binding increased, with little change in the absolute number of counts specifically bound.

Competition studies were conducted as in method 3, using a fixed concentration of [3H]nitrendipine, approximately 700 pM, and ascending concentrations of unlabeled competitor. Nitrendipine and nifedipine were initially diluted in absolute ethanol to make a 1 mM solution before further dilution in 0.9% NaCl. Verapamil and diltiazem were diluted in 0.9% NaCl.

[3H]Nitrendipine binding in myocytes was examined over a range of [K+]o, from 2.4–54.1 mM, and over a range of concentrations of aconitine, 1–300 μg/ml. Aconitine was dissolved in absolute ethanol in a concentration of 3 mg/ml before further dilution in buffer with [K+]o of 5.6 mM. To create a range of potassium concentrations, we used buffer containing [K+]o of 2.5 mM and combined varying volumes of two buffers containing [K+]o of 5.6 and 80 mM in flasks. Aliquots of cells then were added to the flasks containing each concentration of potassium or aconitine, with final cell concentration 2–5 mg wet weight per ml in a final volume of 3 ml. From these flasks, 400-μl cell samples were incubated in triplicate with [3H]nitrendipine, 700 pM, in the absence and presence of unlabeled nitrendipine, 1 μM, as in method 3. To terminate the reaction, 10 ml buffer with the identical [K+]o or aconitine concentration was added, and 15 minutes later the 10-ml volume was filtered over Whatman GF/C glass fiber filters and washed three times with 5 ml of buffer.

For reversibility studies, the cell preparation was divided, and cells were suspended at 25 mg wet weight per ml in approximately 2 ml of buffer containing [K+]o of either 5.6 or 50 mM. They were incubated with a free [3H]nitrendipine concentration of approximately 600 pM, in the absence and presence of unlabeled nitrendipine, 1 μM. After 30 minutes, 500-μl aliquots of cells were removed and diluted in 5 ml of the buffer with either the identical or opposite [K+]o, with [3H]nitrendipine and unlabeled nitrendipine concentrations maintained as in the first incubation. These suspensions were incubated at 37°C, and at various time points triplicate 200-μl aliquots were removed and immersed in 5 ml of buffer, 4°C, having the same [K+]o, as the second incubation. After 15 minutes, these 5-ml volumes were filtered and washed 3 times with 5 ml of buffer, 4°C.

Kinetic Studies

Preliminary studies showed that association and dissociation were complete within 10 minutes. Association time course studies were initiated with the addition of [3H]nitrendipine following 5 minutes of incubation at 37°C. Triplicate 200-μl aliquots were obtained at various times, and each was immersed in 5 ml of buffer at 4°C for 15 minutes before rapid filtration and washing with three 5-ml aliquots of buffer. Nonspecific binding was determined by parallel incubation which included unlabeled nitrendipine, 1 μM. To determine the dissociation time course, cells were incubated 1 hour with [3H]nitrendipine, triplicate 200-μl aliquots were taken for total binding at time 0, then unlabeled nitrendipine was added and subsequent triplicate 200-μl aliquots were obtained at various times.
Membrane Saturation Studies

The experiments were performed in Tris-HCl buffer containing compounds of the following millimolar concentrations: Trizma base, 50; MgCl₂, 9; with concentrated HCl added to yield pH 7.5. In addition, 20 mM MOPS buffer (as described above) was used. Membrane vesicles in concentrations of 13–15 μg protein/ml were incubated in 5-ml volumes of buffer in triplicate in the presence of ascending concentrations of [³H]nitrendipine for 45 minutes. Each incubation volume was rapidly filtered over Whatman GF/C glass fiber filters and washed three times with 5 ml cold buffer. Nonspecific binding was defined as that occurring in the presence of 1 μM unlabeled nitrendipine.

Filters were placed in scintillation vials, 7 ml of scintillation cocktail (New England Nuclear formula 947 or 963) were added, and the counts per minute were determined in a Beckman LS 7500 scintillation counter at 47% efficiency.

Materials

[³H]Nitrendipine was purchased from New England Nuclear. Crystalline nitrendipine was the gift of the Miles Institute of Preclinical Pharmacology and crystalline nifedipine was the gift of Pfizer Laboratories Division. Verapamil was donated by Knoll Pharmaceutical Company, and diltiazem by Marion Laboratories, Inc. Aconitine, MOPS free acid, and Trizma base were purchased from Sigma Chemical Co., HEPES free acid was purchased from Calbiochem-Behring, and all other chemicals used were of highest reagent grade commercially available.

Calculations

Saturation data were subjected to Scatchard analysis (Scatchard, 1949), and linear regression was performed to yield the equilibrium dissociation constant, Kᵣ, and maximum receptor concentration, Bₘ. Cell density was determined by measurement of dry weight and conversion to wet weight as previously described (Farmer, 1983). Bₘ in picomoles per liter was then converted to density per mg wet weight of cells. Analysis of time courses and competition data was done using standard equations (Manalan et al., 1981; Fairhurst et al., 1983; Cheng and Prusoff, 1973):

\[
\ln \frac{[RL]_{eq}}{[RL]_0} = K_{OBS} \frac{R}{F} = -K_{OBS} (1)
\]

\[
K_{OBS} = K_0 [L] + K_{-1} (2)
\]

\[
K_{-1} = \frac{0.693}{T_0} (3)
\]

\[
K_0 = \frac{IC_{50} [L]}{1 + \frac{[L]}{K_0}} (4)
\]

Paired t-tests were used to compare time course and equilibrium data in polarized and depolarized cells using STATPAK (Western Michigan University) on a PDP-11 computer (Digital Equipment Corporation). Comparison of competition data in polarized and depolarized cells was done with one-way analysis of variance of repeated observations. Comparison of binding in cells after sequential incubation in two [K⁺]₀ was done using the Bonferroni t-test.

Results

The myocyte preparation yielded adult rat ventricular myocytes which had initial viability greater than 75% as assessed by rod-shaped morphology criteria. This has correlated well with a low rate of succinate oxidation in the absence of digitonin (Farmer et al., 1983). The cells tolerated extracellular calcium concentration, [Ca²⁺]₀, of 1.8 mM. Viability remained 61 ± 2% (mean ± se) at the conclusion of each experiment. These adult cells were used the same day for experimentation. The isolated cells did not spontaneously contract either in [K⁺]₀ of 5.6 or 50 mM. We have concluded from biochemical data previously described (Farmer et al., 1983) that the cells have impermeable membranes and therefore that varying [K⁺]₀ above 5.6 mM should cause depolarization of the cells.

Figure 1 depicts results from one representative [³H]nitrendipine saturation experiment in polarized and depolarized cells. Specific binding was saturable and ranged from 48 ± 2.5% to 33 ± 2.5% (mean ± se) in polarized cells and 65 ± 1.8% to 45 ± 2.9% in depolarized cells. In the inset is a Scatchard analysis of the two saturation curves. In polarized myocytes, Kᵣ was 962 pm and Bₘ was 44.3 pm or 13.6 fmol/mg wet weight. In depolarized myocytes (○), Kᵣ was 845 pm and Bₘ was 131 pm or 40.1 fmol/mg wet weight.
wet weight. In depolarized myocytes, $K_D$ was 845 pm and $B_{\text{max}}$ 131 pm or 40.1 fmol/mg wet weight. Although the $K_D$ was not significantly changed in the depolarized myocytes, the $B_{\text{max}}$ increased strikingly. Results from 18 experiments in live myocytes are shown in Table 1. In polarized myocytes, the mean $K_D$ was 587 ± 50 pm and $B_{\text{max}}$ was 10.8 ± 1.3 fmol/mg wet weight. In depolarized myocytes, the mean $K_D$ was 661 ± 77 pm and $B_{\text{max}}$ was 25.6 ± 3.7 fmol/mg wet weight. There was no significant difference between the $K_D$ in polarized and depolarized myocytes, but the increase in $B_{\text{max}}$ in depolarized myocytes was highly significant ($P < 0.0001$) by paired $t$-test.

If this phenomenon of increased number of binding sites is related to depolarization of membranes, then cells which have no resting potential should not exhibit changes in binding characteristics when extracellular potassium concentration is changed. We examined dead cell preparations as assessed by morphological evidence of 100% irreversible contraction. These results are also shown in Table 1. In $[K^+]_o$ of 5.6 mm, $K_D$ was 543 ± 126 pm and $B_{\text{max}}$ 17.8 ± 3.4 fmol/mg wet weight. In $[K^+]_o$ of 50 mm, these values were unchanged: $K_D$ was 565 ± 173 pm, and $B_{\text{max}}$ 19.2 ± 3.9 fmol/mg wet weight. We also performed a parallel experiment in which we incubated canine myocardial membrane vesicles in each MOPS buffer. In $[K^+]_o$ of 5.6 mm, $K_D$ was 483 pm with $B_{\text{max}}$ 857 fmol/mg protein and in $[K^+]_o$ of 50 mm, $K_D$ 389 pm and $B_{\text{max}}$ 782 fmol/mg protein. These results were comparable to our results in membrane vesicles incubated in Tris-Cl buffer, in which we observed $K_D$ of 314 ± 28 pm and $B_{\text{max}}$ 823 ± 82 fmol/mg protein.

We performed time course studies of $[3H]$nitrendipine binding to myocytes in both $[K^+]_o$. We found association and dissociation to be monophasic and complete within 5 minutes at 37°C. In polarized myocytes, $K_t$ was 9.725 ± 1.7 × 10^8 M/min and $K_t$ was 0.32 ± 0.04/min. The mean $K_D$ from these five experiments was 436 ± 102 pm. In depolarized myocytes, $K_t$ was 12.056 ± 1.9 × 10^8 M/min and $K_t$ was 0.36 ± 0.03/min, with $K_D$ 315 ± 39 pm.

There was no difference in dead myocytes. These $K_D$ are in good agreement with equilibrium determinations of $K_D$.

We wished to see whether $[3H]$nitrendipine binding was maximal at any particular membrane voltage in order to relate binding to channel activation state. We examined changes in $[3H]$nitrendipine binding as a function of increasing $[K^+]_o$ and this is shown in Figure 2. Specifically bound $[3H]$nitrendipine more than doubled as $[K^+]_o$. was increased from 2.4 mm to 54.1 mm. In $[K^+]_o$ of 4.5 mm, $[3H]$nitrendipine binding in 48-hour-old dead myocytes was 44% greater than in live cells from the same preparation, but it was less than that observed in depolarized live cells. Because we were concerned about a possible time-related loss in binding sites, we treated live cells acutely with digitonin, 5 µg/ml, to abolish membrane potential, and compared binding in these cells with live cells from the same preparation. It is apparent, in Figure 2, that digitonin-treated cells bind as much $[3H]$nitrendipine at 4.5 mm $[K^+]_o$ as depolarized live cells, and there is no further change at higher $[K^+]_o$.

To verify further that the increase in $[3H]$nitrendipine-binding sites was due to depolarization, we treated cells in $[K^+]_o$ of 5.6 mm with ascending concentrations of conoicaine, a sodium channel-activating drug (Peper and Trautwein, 1967; Catterall, 1977); these results are shown in Figure 3. As conoicaine concentration was increased from 1–30 µg/ml, $[3H]$nitrendipine specifically bound in the presence of a free $[3H]$nitrendipine concentration of 700 pm increased to 158 ± 6% of control. Again, conoicaine did not change specific binding in dead cells (data not shown). It should be noted that cells exposed to conoicaine, 300 µg/ml, were also exposed to 10% ethanol, which by itself caused a 9% decrease in specific binding below that seen in 5.4 mm $[K^+]_o$ alone.

If the potassium-induced change in $[3H]$nitrendipine receptor density is the result of differences in membrane voltage, then this change should be rapidly reversible if $[K^+]_o$ is changed. The reversibility of this depolarization-induced binding is depicted.
in Table 2. These figures were obtained after 30 minutes of incubation in the second medium. It can be seen that cells initially and subsequently incubated in $[K^+]_0$ of 5.6 mM bound the same amount of $[^3H]$nitrendipine as did cells that were initially incubated in $[K^+]_0$ of 50 mM prior to incubation in $[K^+]_0$ of 5.6 mM. Cells initially incubated in $[K^+]_0$ of 5.6 mM before incubation in $[K^+]_0$ of 50 mM bound the same amount of $[^3H]$nitrendipine as cells incubated in $[K^+]_0$ of 50 mM for both incubations. However, there was a significant difference between the number of $[^3H]$nitrendipine sites in cells finally incubated in $[K^+]_0$ of 50 mM and in those finally incubated in $[K^+]_0$ of 5.6 mM ($P < 0.01$). Because the number of $[^3H]$nitrendipine-binding sites correlated with the $[K^+]_0$ of the second but not the first incubation, it can be concluded that this change in numbers of binding sites is reversible. Subsequently, we examined the time course of the change in $[^3H]$nitrendipine binding after the cells were placed in the second $[K^+]_0$ and found that binding equilibrated within 1 minute (data not shown). Because of technical limitations, earlier time points could not be examined.

We examined competition of $[^3H]$nitrendipine with unlabeled nitrendipine, nifedipine, verapamil, and diltiazem. In Figure 4, we have depicted competition of unlabeled calcium channel antagonists with $[^3H]$nitrendipine in polarized and depolarized cells. There is no difference in the IC$_{50}$ of nitrendipine in the two $[K^+]_0$. It can be observed that the curves are slightly shallow and, indeed, the slope factor for these mean determinations is less than one. We suspect that a second lower affinity site is being revealed. The IC$_{50}$ of nifedipine is slightly but not significantly greater than that observed with nitrendipine, there is no difference in nifedipine competition in polarized and depolarized cells, and the slope factors are also less than one.

Unlabeled verapamil only partially inhibited $[^3H]$
FIGURE 4. Competition of unlabeled nitrendipine, nifedipine, verapamil, and diltiazem with [3H]nitrendipine in polarized and depolarized adult rat cardiac myocytes. Data points represent mean ± se of results from 7–10 experiments with each competitor. Closed symbols represent results in polarized myocytes. Open symbols represent results in depolarized myocytes. ▼ ▼ Nitrendipine, n = 10; □ □ nifedipine, n = 7; ▲ ▲ verapamil, n = 8; ● ○ diltiazem, n = 8.

nitrendipine, and there was sometimes greater inhibition of binding in depolarized than in polarized cells. Overall, the differences in these eight experiments were not significant. Whereas partial inhibition of binding can be seen at concentrations as low as 1 nM, IC50 for these means of eight experiments was approximately 10 μM. In several experiments, partial inhibition was less than 50% at 10-4 M. The mean slope factor for these eight experiments was much less than one.

The competition of diltiazem with [3H]nitrendipine exhibited remarkable variability. In most experiments in both polarized and depolarized myocytes, diltiazem weakly inhibited [3H]nitrendipine binding. In a total of 4 out of 10 experiments, however, we noted an increase of up to 70% in specific binding in polarized but not depolarized myocytes. Because of the inconsistency of this phenomenon in the myocytes, there was no significant difference overall in the ability of diltiazem to inhibit [3H]nitrendipine binding in polarized and depolarized myocytes. Diltiazem inhibited much less than 50% of [3H]nitrendipine binding. The average slope factors and Ki for each competitor are summarized in Table 3.

Discussion

Our experiments document the presence of a high-affinity binding site for nitrendipine in both polarized and depolarized myocytes. In membranes, the Ko we observed ranged between 314 and 483 pm, in good agreement with the results obtained by others (Glossman et al., 1982; Murphy and Snyder, 1982; Ehler et al., 1982a, 1982b; Bolger et al., 1982; DePover et al., 1982). The Ko we observed in cells was slightly higher, comparable to that reported by DePover (DePover et al., 1983a). The possibility of a second site of lower affinity and higher capacity has previously been raised, both in work in membranes (Bellman et al., 1981; Vaghy et al., 1984), and in cells (Marsh et al., 1983). We have chosen to confine our inquiry to the high-affinity site.

We have observed, for the first time, a significant increase in the number of [3H]nitrendipine-binding sites with depolarization of live cells. This can be demonstrated not only as a function of potassium concentration, but also as a function of aconitine concentration. It is apparent that this increase in binding sites approaches a maximum in [K+]o of 54.1 mM. In addition, this phenomenon is rapidly reversible with changes in extracellular potassium concentration. These observations are consistent with voltage-dependent binding of [3H]nitrendipine in these cardiac myocytes.

Because nitrendipine is a putative calcium channel label, these voltage-dependent changes in binding may occur as a result of changes in the state of activation of the calcium channel. Previous work in

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<th>Competitor</th>
<th>[K+]o</th>
<th>Ki (nM)</th>
<th>Slope factor</th>
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<tbody>
<tr>
<td>Nitrendipine (n = 10)</td>
<td>5.6</td>
<td>2.0 ± 0.35</td>
<td>0.58 ± 0.07</td>
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<tr>
<td></td>
<td>50</td>
<td>1.9 ± 0.47</td>
<td>0.73 ± 0.11</td>
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<tr>
<td>Nifedipine (n = 7)</td>
<td>5.6</td>
<td>6.0 ± 1.7</td>
<td>0.62 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>4.8 ± 1.0</td>
<td>0.76 ± 0.09</td>
</tr>
<tr>
<td>Verapamil (n = 8)</td>
<td>5.6</td>
<td>8317 ± 2580</td>
<td>0.22 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>2339 ± 889</td>
<td>0.21 ± 0.03</td>
</tr>
<tr>
<td>Diltiazem (n = 8)</td>
<td>5.6</td>
<td>Inhibition less than 50%</td>
<td>-0.0305 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0.067 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as mean ± se.
isolated myocytes suggested that the channel exists in either a fully open or possibly two closed states, and that opening occurs in bursts (Reuter, 1982). The probability that channel opening will occur increases with depolarization (Reuter, 1982). Recent observations of an increase in the apparent rate of calcium channel inactivation during the first membrane depolarization in the presence of nitrendipine suggested that nitrendipine binds to the channel in the open state (Lee and Tsien, 1983; Sanguinetti and Kass, 1984). Subsequently, voltage-dependent effects of nisoldipine, nitrendipine, and nicardipine have been noted, and these results suggested that nitrendipine binds predominantly to inactivated channels (Sanguinetti and Kass, 1984). Moreover, recent studies of nifedipine in frog ventricular muscle have suggested that once depolarization of the membrane occurs, photoactivation of nifedipine does not result in unblocking of the channel until a brief period of repolarization occurs (Morad et al., 1983). Our data suggest that the number of binding sites of [3H]nitrendipine is greatest under conditions of prolonged depolarization in which a large proportion of calcium channels is inactivated. Taken together, these observations support the concept of state-dependent binding of nitrendipine to the myocardi al calcium channel and indicate that nitrendipine binds not only to open but to inactivated channels, as well.

The binding affinity of nisoldipine and nitrendipine in depolarized myocardium has been predicted to increase by three orders of magnitude over that affinity observed in polarized myocardium (Sanguinetti and Kass, 1984). This observation might help to explain the discrepancy between the $K_D$ of the [3H]nitrendipine receptor in myocardium and the half-maximal concentration, $IC_{50}$, of nitrendipine which produces negative inotropic effects (Millard et al., 1983) and reduces sarcoplasmic calcium influx (Lee and Tsien, 1983). We were unable to demonstrate any difference in $K_D$ between polarized and depolarized cells, however, even though the modulated receptor hypothesis would have predicted a higher $K_D$ in polarized cells. It is possible that occupancy of a low affinity receptor correlates with pharmacological effect (Marsh et al., 1983; Vaghy et al., 1984) or that there is unusual coupling of the pharmacological effect to receptor occupancy. It is also possible that membrane depolarization may open access to a channel-associated receptor without changing receptor affinity (Starmer et al., 1984).

We found that nitrendipine and nifedipine completely inhibited binding of [3H]nitrendipine, with a slope factor less than 1, comparable to cultured cell data (Marsh et al., 1983). We concur that there may be lower affinity sites detected by these competition studies. Our results demonstrate no difference in the potency of these calcium antagonists in polarized vs. depolarized cells. Verapamil only partially inhibited binding, and this is in agreement with observations in membranes (Ehlert et al., 1982a,b). Verapamil was previously reported to have a $K_D$ of 8 nM for a lower affinity site in cells (Marsh et al., 1983), but our observations pertain to the higher affinity site, at which verapamil competed slightly at 1 nM and to a greater extent at micromolar concentrations. Diltiazem weakly competed for [3H]nitrendipine-binding sites, and in only one experiment did this exceed 50% inhibition of specific binding. We observed diltiazem-induced enhancement of [3H]nitrendipine binding at 37°C in polarized but not depolarized myocytes. This phenomenon in membranes has been well described, and has been ascribed to changes in the rate of dissociation of the ligand in brain (Yamamura, 1982; Ferry and Glossman, 1982) and in myocardium (Janis et al., 1984) and to increases in the number of receptors in myocardium (DePover et al., 1982; Millard et al., 1982). Given that membranes have no electrical potential, it is unclear to us why depolarized cells did not exhibit the same enhancement of [3H]nitrendipine binding. Our results on diltiazem-nitrendipine interactions are in contrast with those obtained in embryonic chick ventricular cells, in which no interaction was observed (Marsh et al., 1983).

If we assume $4.4 \times 10^6$ cells per mg wet weight, our maximum nitrendipine receptor density was 160,000 sites per polarized cell and 382,000 sites per depolarized cell. These are greater than estimates of 2,000–10,000 channels/cell based on electrophysiological studies of cultured neonatal rat cardiac cells (Reuter, 1983). One other study in isolated adult rat cardiac myocytes estimated $10^6$ sites/cell (DePover et al., 1983a). Studies in cultured embryonic chick ventricular cells measured 51 fmol/mg protein (Marsh et al., 1983). This can be compared with 61 and 147 fmol/mg protein in these rat heart cells in $[K^+]_o$ of 5.6 and 50 mM, respectively. Discrepancies in binding data may be the result of different methods of converting concentrations of receptors in solution to density per milligram of tissue or number of sites per cell. It is more difficult to reconcile the binding data with the electrophysiological data. It is unlikely that multiple nitrendipine molecules bind to one channel. Our cells are from adult rats and are not cultured. By contrast, electrophysiological estimates were from cultured neonatal rat cells. It is possible that age-related differences in density or binding sites exist. In addition, cultured cells may have binding characteristics different from those of freshly isolated cells that have been recently exposed to enzymatic digestion (Glossman et al., 1982).

Observations in subcellular fractions of myocytes also provide a possible explanation for the increase in the number of [3H]nitrendipine-binding sites in depolarized myocytes. Subfractions of cardiac sarcoplasmic reticulum have been isolated by selective calcium oxalate loading and discontinuous sucrose density gradient centrifugation (Jones and Cala, 1981). Three fractions which demonstrate increased calcium uptake in the presence of the alkaloid ry-anodine have particularly high concentrations of
[3H]Nitrendipine-binding sites (Williams and Jones, 1983), and these findings have subsequently been confirmed in a different preparation technique (DePover et al., 1983a). Thus, there may be intracellular binding sites for [3H]Nitrendipine which are not detected by electrophysiological study (DePover et al., 1983a).

Although [3H]Nitrendipine binding sites do not copurify with sarcomemmal markers such as Na+, K+-ATPase, and muscarinic cholinergic and β-adrenergic receptors, the cellular location of [3H]Nitrendipine-binding sites is still unclear. The ryanodine-sensitive fraction of sarcoplasmic reticulum that is collected at the 1.0 M:1.5 M sucrose interface has high concentrations of a 55,000-dalton calcium-binding protein (Jones and Cala, 1981; Campbell et al., 1983). This protein cross-reacts with antiserum to calsequestrin, a skeletal muscle protein localized to terminal cisternae (Jorgensen et al., 1982; Campbell et al., 1983). There is evidence that this cardiac calsequestrin is also located in junctional sarcoplasmic reticulum (Campbell et al., 1983; Jorgensen et al., 1984). It is possible that this membrane fraction contains dyads and triads of sarcomemmal T-tubules and sarcoplasmic reticulum. It is also possible that there are calcium release channels in sarcoplasmic reticulum membranes which are detected as nitrendipine-binding sites. Recently, a dihydro-pyridine analog has been used as a photoaffinity label of a 32,000-dalton membrane protein found to be enriched in ryanodine-sensitive membrane fragments from canine myocardium (Campbell et al., 1984). Similar studies have isolated a 41,000-dalton protein in intestinal smooth muscle membrane (Venter et al., 1983). These studies may aid future attempts to localize these binding sites within the myocardial cell.

High levels of nonspecific binding remain a problem for cellular binding studies using [3H]Nitrendipine. It is interesting that multiple washings of isolated cells with consequent removal of albumin have been observed to reduce nonspecific binding (DePover et al., 1983). We have had improved results by allowing our samples to equilibrate in a large volume of ice cold buffer for 15 minutes. Time course studies have indicated that this reduces total and nonspecific binding without changing specific binding. High nonspecific binding still hampers investigation of a possible second lower affinity binding site (DePover et al., 1983a).

Our results suggest that radioligand-binding studies in cells may be of value in correlating alterations in nitrendipine binding with manipulations that are known to modulate the calcium channel. Contrary to hypotheses generated from electrophysiological studies, we did not detect a difference in the affinity of receptor sites in polarized and depolarized cells, but depolarization did increase the number of nitrendipine-binding sites. Our estimates of receptor density, taken together with others, suggest that there may be more [3H]Nitrendipine-binding sites per cell than sarcomemmal calcium channels, as predicted by electrophysiological measurements of sarcomemmal calcium current.

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Address for reprints: Frank J. Green, M.D., Krannert Institute of Cardiology, 1001 West 10th Street, Indianapolis, Indiana 46202.

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