Evidence for Distinct Calcium Channel Agonist and Antagonist Binding Sites in Intact Cultured Embryonic Chick Ventricular Cells

Richard T. Lee, Thomas W. Smith, and James D. Marsh

To determine whether calcium channel agonists and antagonists bind to distinct pharmacologically active sites, the binding of dihydropyridine calcium channel agonists and antagonists was related to calcium flux and contractile state in primary monolayer cultures of spontaneously contracting chick embryo ventricular cells. Equilibrium binding studies using the antagonist (+)-[3H]PN200-110 demonstrated equilibrium binding to intact, beating cells consistent with a single class of binding sites (K_D, 1.1 nM; B_max, 40 fmol/mg protein). Membrane depolarization of the intact cells by incubation in 30 mM potassium caused a 91% increase in the apparent number of (+)-PN200-110 binding sites (B_max 76 fmol/mg protein), but no significant change in the K_D (1.2 nM). The (+)-PN200-110 produced a concentration-dependent decrease in calcium uptake (IC_50 2.2 nM) and contractile amplitude (IC_50 5.6 nM). The calcium channel agonist, (±)-[3H]Bay 8644, bound to two distinct binding sites with high affinity (K_D 1.0 nM) and low affinity (K_D 1.9 µM). The (±)-Bay k 8644 produced biphasic modulation of calcium flux and contractile state. At concentrations of 100 nM or less, (±)-Bay k 8644 increased calcium flux and contractile amplitude, consistent with drug interaction with the high affinity agonist site. However, at higher concentrations, the stimulatory effect of (±)-Bay k 8644 on calcium flux and contractile amplitude was abolished, a finding that is consistent with drug interaction with the low affinity antagonist site. Ligand binding studies using (±)-Bay k 8644 to displace (+)-[3H]PN200-110 110 demonstrated that these ligands compete at a single site with K_D for (±)-Bay k 8644 of 1.7 µM, which is consistent with binding at the low-affinity antagonist site. It was concluded that in intact cultured myocytes, voltage-sensitive calcium channels have at least 2 distinct dihydropyridine binding sites.

Binding of dihydropyridines to these sites mediates opposing effects on calcium flux and contractile function. (Circulation Research 1987;60:683–691)

Transsarcolemmal influx of calcium via slow calcium channels is essential for excitation-contraction coupling in cardiac muscle.1–2 There is electrophysiologic evidence that calcium channel function can be modulated by calcium channel agonists as well as by calcium channel antagonists.3 The most potent class of calcium channel modulators, the dihydropyridines, includes antagonists such as nifedipine and a relatively new agent, (+)-PN200-110 [isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxy-carbamyl-2,6-dimethyl-3-pyridinecarboxylate].4,5 Structural modification of dihydropyridine antagonists has yielded several calcium channel agonist compounds, such as CGP 28392 (4-[2-(difluoromethoxy)phenyl]-1,4,5,7-tetrahydro-2-methyl-5-oxo-fluoro[3,4-b]pyridine-3-carboxylic acid ethylester) and (±)-Bay k 8644 (1,4-dihydro-2,6-dimethyl-3-nitro-4-(2-trifluoromethylphenyl)-pyridine-5-carboxylate) that increase inward current through the calcium channel.6–9

Electrophysiologic studies have defined at least two different inward calcium currents in excitable cells.6–11 These calcium currents differ in their responses to dihydropyridines; proteins that modulate these currents may represent products of different genes. The channel mediating a fast calcium current probably possesses only a low-affinity dihydropyridine binding site. In some cells, this fast channel is unlikely to be important for electrical excitability.11 However, the channel that mediates the slow calcium current can respond to nitrendipine with both stimulation and inhibition of calcium currents. To account for these complex effects, Bean has suggested that the slow calcium channel may have more than one dihydropyridine binding site.11

Previous studies in our laboratory using radioligand binding techniques have identified two binding sites of different affinity for nitrendipine in intact cultured chick myocardial cells.12 Two binding sites or states were subsequently reported in canine sarcolemma13 and chick heart membranes.14 Because nitrendipine exerts its typical effects at a concentration near the K_D of the low-affinity site, it has been proposed that the binding of antagonists to the low-affinity site mediates antagonism of calcium influx.12 The physiologic or pharmacologic significance of the high-affinity binding site is uncertain. Sanguinetti and Kass15 have provided electrophysiologic evidence that interaction of dihy-

From the Cardiovascular Division, Departments of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, Mass.

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Address for correspondence: Dr. James D. Marsh, Cardiovascular Division, Brigham and Women’s Hospital, 75 Francis Street, Boston, MA 02115.

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dihydropyridines is 1,000 times stronger with inactivated channels than with resting channels. Green et al. have observed that there is an apparent increase in the number of high-affinity nitrendipine binding sites in response to potassium-induced membrane depolarization. The physiologic significance of greater expression of high-affinity binding remains unclear however.

While it has been established that depending on the preparation and the ligand, antagonists may bind to one or two sites (or states) of the calcium channel, less is known about binding properties of dihydropyridine calcium channel agonists. Janis et al. have reported that the agonist BAY k 8644 has radioligand binding properties consistent with a two-site model in a rabbit cardiac membrane system. It has been proposed that the high-affinity BAY k 8644 binding site may be the binding site mediating an increase in calcium channel openings, while the low-affinity site may lead to a decrease in channel openings.

To investigate further the relations of dihydropyridine antagonist and agonist binding to physiologic function of calcium channels, binding and functional response to the antagonist (+)-PN200-110 and the agonist (±)-BAY k 8644 were studied in intact cultured heart cells. This experimental model allowed detailed examination of radioligand binding properties, calcium flux measurements, and contractile effects under virtually identical experimental conditions.

The following related questions were addressed: 1) Do radioligand binding studies of (+)-PN200-110 and (±)-BAY k 8644 identify specific binding sites, occupancy of which modulates function? 2) What is the relation between agonist and antagonist binding sites in intact, viable cells? 3) Do binding properties of (+)-[3H]PN200-110 and (±)-[3H]BAY k 8644 change with membrane depolarization? It is possible that pharmacologically defined binding sites reflect different biophysical states of the calcium channel or different calcium channels. For the sake of clarity, the convention of describing pharmacologic binding sites will be observed. Our findings indicate that binding of a dihydropyridine agonist to one site of the channel leads to an increase in calcium flux and augmented contractile state, whereas binding to a second site can lead to a decrease in calcium flux and diminished contractile state.

Materials and Methods

Tissue Culture

Primary monolayer cultures of spontaneously beating chick embryo ventricular cells were prepared as previously described. Briefly, fragments of embryonic chick ventricles 10 days in ovo were dissociated by repeated cycles of trypsinization. The resulting cell suspensions were plated in 35-mm multiwell culture dishes and grown in a culture medium consisting of bicarbonate-buffered physiologic salt solution containing 40% medium 199 (Gibco, Grand Island, N.Y.), 6% fetal calf serum (Gibco), and 54% balanced salt solution containing glucose. Final mM concentrations in the culture medium were Na+ 144, K+ 4.0, Ca++ 0.97, HCO3− 18, Mg++ 0.8, and Cl− 131. Cultures were incubated in a humidified 5% CO2−95% air atmosphere at 37°C. Spontaneously and synchronously contracting confluent monolayers were present by 3 days in culture. For contractility and ion flux experiments, cells were grown on 25-mm circular glass coverslips.

Contractile Response Measurements

To determine the contractile response of cultured heart cells to (±)-BAY k 8644 and (+)-PN200-110, measurements of the amplitude of contraction of individual cells in a spontaneously contracting monolayer at 3 days in culture were conducted with the phase contrast microscope-video motion detector system previously described in detail. The medium that superfused cells during contractility measurements was a HEPES-buffered solution containing (in mM) HEPES (N-2-hydroxyethylpiperazine-N-2-ethano sulfonic acid) 5.0, CaCl2 0.9, KCl 4.0, NaCl 140, MgCl2 0.5, and 2% fetal calf serum. Flow rate was 2 ml/min. Following a 10-minute equilibration period, cells were superfused with varying concentrations of (±)-BAY k 8644 or (+)-PN200-110. For experiments using (±)-BAY k 8644, the response was compared with the contractile response to HEPES-buffered solution containing 3.6 mM Ca++, a stimulus that produces maximal contractile response in this system. The amplitude of contraction was recorded and measured as described by Marsh et al. Previous studies from our own and other laboratories have demonstrated that changes in amplitude and velocity of wall motion can be used to quantify the effects of a variety of interventions on cell contractility.

Loading conditions for contraction caused by attachment of the cell to the coverslip and other cells, as well as the viscoelastic properties of each cell, may vary from cell to cell. However, by expressing the contractile response of the cell as a percentage of its maximal response, these differences in loading conditions are taken into account, and each cell can serve as its own control. Previous studies from this laboratory have demonstrated that this method of assessing the inotropic state of cells in monolayer culture correlates closely with other physiologic parameters expected to relate to inotropic state, such as alterations in monovalent and divalent cation fluxes and response to altered oxygen tension. The contractility measurements were made on only 1 cell per coverslip. Several coverslips were used from 1 plating; all experimental points comprise data from 2 or more platings.

Radioligand Binding Experiments

For determination of equilibrium binding properties of (+)-[3H]PN200-110 and (±)-[3H]BAY k 8644, monolayers of cultured ventricular cells were washed twice in 37°C HEPES buffer with a mM concentration of CaCl2 0.6, HEPES 5.0, KCl 4.0, NaCl 140, MgCl2 0.5, and pH 7.35. Graded concentrations of radioligand and unlabelled dihydropyridine were added and
the monolayer was then incubated at 37°C for 1 hour. Unbound (+)-[3H]PN200-110 was removed by washing the monolayer twice with HEPES buffer at 37°C; cells were removed from the substrate by treatment with 0.04% trypsin at 37°C for 30 minutes. Aliquots of cell suspensions were counted at 22% efficiency in a Packard liquid scintillation counter (Packard Instrument Co., Downers Grove, III.). For experiments using (±)-[3H] BAY k 8644, at the conclusion of binding at 37°C, a 5-minute incubation in 0°C HEPES buffer was used to reduce nonspecific binding. It had been determined that this step was optimal for removing nonspecific binding at concentrations of (±)-[3H] BAY k 8644 of 1 nM or greater, with no effect on specific binding. The monolayer was then washed twice in 0°C HEPES buffer, and cells were recovered as described above. For experiments on the effects of membrane depolarization, the concentration of potassium in the HEPES buffer was 30 mM, both in the media for the binding reaction and for the wash step, with the sodium concentration reduced to 100 mM. Cells were no longer spontaneously contractile in 30 mM potassium.

Equilibrium binding data and displacement curves were analyzed by the iterative, nonlinear least squares method of Munson and Rodbard24 using a DEC 11/23 computer. This program solves the system of multiple linear equations describing the interaction between ligand L, and receptor R, with equilibrium constant KLR. It incorporates an additional term for nonspecific binding, N. Because the program uses as input only the untransformed experimental data, total ligand concentration, and bound ligand concentration, analysis is not confounded by reduction in free ligand concentration by internalization or high receptor concentration, as may be the case for conventional Scatchard analysis. Simplifying assumptions further to relate IC50 to Kd by the commonly used Cheng-Prusoff correction are also unnecessary since the Kd for each effect is calculated directly. To test the hypothesis that ligand displacement curves are better described by a one-site than a two-site model, Kd for a ligand was initially estimated as the value determined by analysis of equilibrium binding isotherms, and the system of equations was solved for the number of receptors and for nonspecific binding. The equations were then solved with an additional binding affinity and variable receptor number included in the model; Kd was also allowed to vary. An F test was used to compare goodness of fit for the two models. If the two-site model described the data better at the p<0.05 level, then the one-site hypothesis was rejected.

Each point was assayed in triplicate, and each individual binding curve was replicated at least 3 times. All equilibrium dissociation constants are expressed as the mean ± SD.

**Calcium Flux Measurement**

The procedures for measuring calcium fluxes have been described in detail by Biedert et al19 and Barry and Smith.22 For determination of calcium uptake, cells were first incubated in zero calcium HEPES buffer with the dihydropyridine for 5 minutes. This preincubation step increases the 45Ca specific activity at the sarcolemmal boundary available for influx through the slow calcium channel (D. H. Kim, unpublished observations). Initial experiments indicated that (+)-PN200-110 and (±)-BAY k 8644 at the concentrations used reached equilibrium binding in 5 minutes.

The cells were then transferred to medium containing the same concentration of the dihydropyridine and 45Ca (5 μCi/ml; [Ca]-0.9 mM) for 30 seconds. The t0 for equilibration of the rapidly exchangeable calcium pool in this experimental protocol is 11 seconds.19 Therefore, the 30-second incubation in 45Ca labels greater than 85% of the rapidly exchangeable calcium pool without an appreciable contribution from the slowly exchangeable pool. Cells were washed twice for 8 seconds each by gently agitating the glass coverslips sequentially in two 60-ml volumes of ice-cold balanced salt solution. Cells were then dissolved in medium containing 1% sodium dodecyl sulfate and 10 mM sodium borate and the aliquots assayed for radioactivity.

**Materials**

The (+) enantiomer of [-3H] PN200-110 was obtained from Amersham, Arlington Heights, Ill. 45Ca and (±)-[3H] BAY k 8644 was obtained from New England Nuclear, Boston, Mass. Miles Laboratories, West Haven, Conn., kindly supplied (±)-BAY k 8644 and the negative enantiomer, BAY R 5417, and Sandoz, Basel, Switzerland, the (+) enantiomer of PN200-110. Other reagents were from Fisher Scientific, Springfield, N.J.

Dihydropyridines were dissolved in 70% ethanol at a concentration of 10^-2 M for BAY k 8644 and 10^-3 for PN200-110, protected from light, stored a maximum of 3 weeks, and subsequently diluted in buffer prior to use. Control experiments confirmed that the low final ethanol concentration by itself had no measurable effect.

**Results**

**Binding Studies With (+)-PN200-110**

To characterize the binding of (+)-PN200-110 to intact myocardial cells, binding isotherms were determined. Over the concentration range studied, binding reached equilibrium in 5 minutes. To test the hypothesis that membrane depolarization increases the number of available dihydropyridine antagonist binding sites, isotherms were also determined in medium containing 30 mM KCl. At each concentration, membrane depolarization led to a significant increase in binding of (+)-[3H] PN200-110. Specific binding was at least 70% of total binding at concentrations of 400 PM or less of (+)-[3H] PN 200-110. However, at concentrations of radioligand greater than 1 nM, specific binding declined to <20% of total binding. Under these conditions, traditional Scatchard analysis can be misleading, and there may be substantial error in estimates of Kd and Bmax.23 Therefore, (+)-[3H] PN200-110 binding...
ing studies were performed with varying concentrations of unlabelled (+)-PN200-110 to displace a constant concentration of radioligand (Figure 1). These competition binding studies also offered the advantage of improved resolution of affinity states of the receptor. Displacement studies performed on spontaneously contracting cells in the presence of control buffer (4 mM potassium) yielded an equilibrium binding constant \( K_D \) of 1.1 ± 0.2 nM \((n = 6)\), with a \( B_{\text{max}} \) of 40 ± 7.6 fmol/mg protein (mean ± SD). The mean non-specific binding for these experiments was 47%. In each case, the fit of a two-site model for (+)-PN200-110 binding was not statistically better than the fit for a one-site model.

To confirm that membrane depolarization alters binding of (+)-[\( ^3 \)H]PN200-110, competition binding studies on potassium-depolarized cells were performed simultaneously on parallel cultures. These studies demonstrated no significant change in the \( K_D \) (1.2 ± 0.2 nM), but the number of receptor sites was significantly increased by a factor of nearly two \( (B_{\text{max}} = 76 ± 16 \, \text{fmol/mg protein}, \, p < 0.05)\). The slope factor for these curves was 0.98 ± 0.08, consistent with a single-site model for ligand binding. Attempts to fit the data to a two-site model for binding did not improve the goodness of fit. These data demonstrate that the antagonist (+)-[\( ^3 \)H]PN200-110 binds to a single, high-affinity receptor site and that potassium-induced membrane depolarization causes an increase in available (+)-PN200-110 binding sites without a significant change in affinity for the ligand.

Radioligand Binding Studies With (±)-BAY k 8644

To characterize the radioligand binding properties of (±)-BAY k 8644, displacement studies using a fixed concentration of (±)-[\( ^3 \)H]BAY k 8644 \( (1 \, \text{nM})\) and varying concentrations of unlabelled (±)-BAY k 8644 were performed (Figure 2). Binding curves were distinctly biphasic in each of 5 experiments, and a two-site model was preferred over a one-site model \((p < 0.01)\). The equilibrium dissociation constant for the high-affinity site of BAY k 8644 was 1.0 ± 0.45 nM. A second, low-affinity site was clearly seen with \( K_D = 1.9 ± 0.73 \, \mu \text{M} \). In contrast to the (+)-PN200-110 binding data shown above, there was no significant change in (±)-[\( ^3 \)H]BAY k 8644 binding following 30 mM potassium depolarization. The \( B_{\text{max}} \) for the high-affinity site was 93 ± 25 fmol/mg and for the low-affinity site 177 ± 66 pmol/mg protein.

To assess the interaction of (±)-BAY k 8644 with the binding site for (+)-PN200-110, competitive displacement studies of (+)-[\( ^3 \)H]PN200-110 with varying concentrations of unlabelled (±)-BAY k 8644 were performed (Figure 3). Nonspecific binding in these experiments averaged 29 ± 5% of total binding. The (±)-BAY k 8644 displaced (+)-[\( ^3 \)H]PN200-110
with an equilibrium dissociation constant for (+)-BAY k 8644 of 1.7 ± 0.3 μM, a concentration 2 orders of magnitude greater than the concentration of (+)-BAY k 8644 required for the expression of substantial calcium channel agonist activity.3 The slope factor of this curve was 1.2 ± 0.1, consistent with the view that (+)-BAY k 8644 and (+)-PN200-110 compete for the same single site at micromolar concentrations of (+)-BAY k 8644.

When these displacement studies were performed in the presence of 30 mM potassium, a consistent and statistically significant decrease in the equilibrium dissociation constant for (+)-BAY k 8644 was detected (K₀ 0.3 ± 0.1 μM, p < 0.05). The experimental conditions were identical to those yielding the finding of no decrease in K₀ of (+)-PN200-110 in response to potassium-induced depolarization. Therefore, the (+)-PN200-110 binding site may undergo subtle changes during depolarization that may be apparent with some dihydropyridines but not with others.

**Contractile Effects of Dihydropyridines**

To assess the influence of (+)-PN200-110 on myocardial inotropic state, the contractile response of cultured myocardial cell monolayers was studied. The cardiac inotropic state, the contractile response of cultured heart cells to graded concentrations of (+)-PN200-110 produces an increase in contractile amplitude in a concentration-dependent manner (Figure 4). At no concentration did (+)-BAY k 8644 produce an increase in contractile amplitude or a change in beating rate. Inhibition of contractile amplitude was complete in 5 minutes (consonant with binding kinetics) and was fully reversed 20 minutes after drug removal. Log-logit transformation of the concentration-effect curve yielded an IC₅₀ for altered contractility of 2.2 nM, in reasonable agreement with the K₀ determined by (+)-[³H] PN200-110 binding studies (K₀ 1.1 nM). These data suggest that the single high-affinity binding site for (+)-PN200-110 is pharmacologically relevant and that occupancy of this site leads to a decrease in contractility. This finding is in contrast to previous studies with nitrendipine, which appears to bind to 2 sites with no evident pharmacologic effect attributable to the occupancy of the high affinity site.¹²

To determine the effects of the agonist (+)-BAY k 8644 on contractility, measurements were then performed using graded concentrations of (+)-BAY k 8644 (Figure 5). In these experiments, only cells that responded to 3.6 mM calcium with an increase in amplitude of contraction of 80 to 100% were used; this criterion tends to minimize cell-to-cell variability. The (+)-BAY k 8644 did not change beating rates. Although (+)-BAY k 8644 is commonly considered a pure calcium channel agonist, a biphasic concentration-effect curve was found in these experiments. The EC₅₀ for the positive inotropic effect of (+)-BAY k 8644 was 20 nM. At concentrations greater than 100 nM, the positive inotropic effect of (+)-BAY k 8644 diminished; on average, (+)-BAY k 8644 decreased the amplitude of contraction of cells below control at a concentration of 10 μM. Higher concentrations were not examined in detail because of interference from the alcohol solvent of (+)-BAY k 8644. These experiments indicate that the effect of (+)-BAY k 8644 is more complex than simply a calcium channel agonist effect on myocardial cells and that a second effect, possibly due to calcium channel antagonism, is seen at higher concentrations. The negative inotropic effect occurs at concentrations near the K₀ of the low-affinity binding site of (+)-BAY k 8644 (K₀ 1.7 μM in (+)-[³H] PN200-110 displacement studies; K₀ 1.9 μM in (+)-[³H] BAY k 8644 displacement studies). Therefore, at high concentrations (1 μM), (+)-BAY k 8644 appears to bind to the (+)-PN200-110 antagonist binding site and produces a negative inotropic effect. At lower concentrations, (+)-BAY k 8644 binds predominantly to a high-affinity site and causes a positive inotropic effect.

To determine whether the biphasic contractile response could be attributed to the 2 stereoisomers of BAY k 8644 having opposing effects, contractile response studies were repeated using the pure levorotatory enantiomer of BAY k 8644, (-)-BAY R 5417. At 10⁻⁻⁷ M (-)-BAY R 5417, there was a mean 65% positive contractile response, while at 1 × 10⁻⁵ M, the

![Figure 4](image-url)  
**Figure 4.** Concentration-effect curve for contractile response of cultured heart cells to graded concentrations of (+)-PN200-110. Each point is mean ± SD of at least 5 individual measurements. At concentrations of (+)-PN200-110 less than 10⁻¹⁰ M, no effect was observed.

![Figure 5](image-url)  
**Figure 5.** Concentration-effect curve for contractile response to BAY k 8644. Percent maximal amplitude of contraction (100%, response to 3.6 mM Ca⁺⁺ for ± BAY k 8644) is plotted as a function of drug concentration. Each point is mean ± SD of at least 6 determinations for ± BAY k 8644. O, contractile response to pure negative enantiomer of BAY k 8644 (n = 3–7).
contractile response was +22%. Thus, the pure levorotatory enantiomer produced a clear-cut biphasic response similar to that for the racemic mixture.

**Modulation of 45Ca Influx by Calcium Channel Agonists and Antagonists**

To determine whether the inotropic effects of (+)-PN200-110 and (±)-BAY k 8644 are mediated by altered transsarcolemmal calcium flux, the uptake of 45Ca in response to graded concentrations of the dihydropyridines was studied (Figure 6). The (+)-PN200-110 decreased 45Ca uptake in a concentration-dependent manner with \( IC_{50} = 4.4 \, \text{nM} \), in reasonable agreement with the findings from contractility studies (IC\(_{50} = 2.2 \, \text{nM} \)) and radioligand binding studies (K\(_{D} = 1.1 \, \text{nM} \)). In contrast, (±)-BAY k 8644 (100 nM) increased 45Ca uptake by 85%. The maximal stimulation of 45Ca uptake occurred at the same (±)-BAY k 8644 concentration that elicited maximal positive inotropic effect. The EC\(_{50} \) for (±)-BAY k 8644 in both the contractility studies (20 nM) and the 45Ca uptake studies (20 nM) was substantially greater than the K\(_{D} \) determined by radioligand binding analysis for the high-affinity site (1.0 nM). This finding suggests that near saturation of high-affinity sites by the ligand is necessary to mediate increased calcium flux or that a lower affinity site is present that was not apparent in the radioligand binding studies. At concentrations greater than 100 nM, the increase in 45Ca uptake stimulated by (±)-BAY k 8644 was smaller, and by 10 \( \mu \text{M} \), no statistically significant effect on 45Ca uptake was seen, although there was a trend toward inhibition of calcium uptake. This biphasic modulation of 45Ca uptake closely parallels the biphasic contractile response and supports the hypothesis that (±)-BAY k 8644 has two concentration-dependent opposing effects on calcium channel function.

**Figure 6.** Effect of (+)-PN200-110 and (±)-BAY k 8644 on 45Ca uptake. Cells were incubated in medium containing desired concentration of dihydropyridine and 0 [Ca\(_{0} \) for 5 minutes, and then exposed to medium containing 45Ca for 30 seconds. Values shown are means ± SD for 6 measurements for each point in (±)-BAY k 8644 (○) and (+)-PN200-110 (©). This experiment was repeated 3 times with similar results.

**Discussion**

In this study relating the ligand binding properties of a dihydropyridine antagonist and an agonist to calcium channel function in intact cardiac cells, there are three principal findings. First, the antagonist (+)-PN200-110 binds to a single high affinity site in intact cells with K\(_{D} = 1.0 \, \text{nM} \) and a slope factor near 1. Because the K\(_{D} \) is similar to the concentration of (+)-PN200-110 that significantly reduces 45Ca uptake and contractile state, occupancy of this site by ligand appears to mediate these functionally important effects. Studies of rabbit aorta and guinea pig atria have also found that the concentration of (+)-PN200-110 that causes a 50% decrease in contraction force is close to 1 nM. Second, it was found that in contrast to the antagonist (+)-PN200-110, the agonist (±)-BAY k 8644 binds to 2 sites in the calcium channel. The K\(_{D} \) for the high-affinity (±)-BAY k 8644 binding site (1.0 nM) was similar to the K\(_{D} \) for the high-affinity site reported by Janis et al based on studies with rabbit ventricular microsomes. The high-affinity binding site appears to mediate an increase in 45Ca uptake and a positive inotropic response as well. At higher concentrations, there is a decrease in 45Ca uptake and a progressive decrease in the inotropic response. These effects occur at concentrations close to the K\(_{D} \) for the low-affinity site identified with (±)-[3H] BAY k 8644 (K\(_{D} = 1.9 \, \mu \text{M} \)). Moreover, (±)-BAY k 8644 displaces (+)-[3H] PN200-110 with an apparent K\(_{D} \) for (±)-BAY k 8644 of 1.7 \( \mu \text{M} \), corresponding to occupancy of the low-affinity calcium channel antagonist site. These observations support the hypothesis that the calcium channel possesses 2 separate dihydropyridine binding sites that produce opposite effects on calcium flux. Further supporting the two-site hypothesis is the observation that there is a decrease in the (±)-BAY k 8644 calcium channel agonist effect at micromolar concentrations in isolated rat ventricular strips. In addition, whole-cell clamp calcium current measurements indicate that the number of calcium channel openings decreases at high concentrations of (±)-BAY k 8644. It is unlikely that increased intracellular calcium concentration is leading to calcium channel inactivation. When [Ca\(_{i} \)] is elevated by Na/K ATPase inhibition,
The third principal finding of these studies is that during potassium-induced membrane depolarization, the number of (+)-PN200-110 binding sites increases, while the Kp does not change significantly. The increase parallels an increase in nitrendipine binding sites in depolarized cells reported by Green et al. Binding in the spontaneously beating control cells may reflect an average of dynamically changing dihydropyridine binding sites during depolarization and repolarization; when cells are uniformly depolarized by high potassium, (+)-PN200-110 binding increases.

Our studies of (+)-[3H]PN200-110 displacement by (±)-BAY k 8644 did reveal a fivefold, statistically significant increase in the apparent Kp of (±)-BAY k 8644 (1.7 μM to 0.3 μM) during depolarization. This increase in affinity for the calcium channel agonist is consistent with a preliminary report of another new calcium channel agonist, Sandoz 202-791, which increases its affinity 17-37-fold during potassium-induced membrane depolarization. That the affinity for some dihydropyridines changes during potassium-induced membrane depolarization, while remaining unchanged for others, may reflect subtle changes in conformation of the dihydropyridine binding site during depolarization or a different interaction of different dihydropyridines with 2 separate types of calcium channels.

Our studies with BAY k 8644 revealed a mean binding capacity of 177 pmol/mg protein for the low-affinity site, which is substantially greater than the binding capacity for the high affinity site of BAY k 8644 or for the PN200-110 binding site. Green et al. have noted that dihydropyridine binding data yield greater calcium channel density than that determined by electrophysiologic studies. DePover et al. have studied membrane preparations, have suggested that there may be intracellular binding sites for dihydropyridines. Thus, binding of BAY k 8644 to the low affinity antagonist site may in part represent inactive and/or intracellular calcium channels; the physiologic data indicate that at least some of these binding sites are closely coupled to the sarcolemmal calcium channel.

The findings presented here suggest that the model of the calcium channel containing a single dihydropyridine binding site or state represents an oversimplification. Bean has suggested that a complex model, perhaps with multiple dihydropyridine binding sites, is required to explain the response of the slow calcium channel to dihydropyridines. The calcium channel from skeletal muscle transverse tubules and from chick heart has been reported to comprise 3 proteins, and in most systems studied, there is complex allosteric interaction between the dihydropyridine binding site and the phenylalkylamine binding site. Weiland and Oswald have also reported 2 dihydropyridine binding sites in rat brain membranes.

Thus, our results with depolarization-induced alteration in the number of (+)-PN200-110 binding sites, as well as separate agonist and antagonist binding sites, may be interpreted in at least two different ways. First, there may be two structurally distinct dihydropyridine binding sites present, with one site a calcium channel activator, to which binding increases the probability of channel opening (the agonist site), while another distinct site in this model would be the inactivator, producing decreased probability and/or duration of channel opening. Second, there may be a change in conformation of a structurally homogeneous population of dihydropyridine binding sites by membrane depolarization, with dihydropyridine antagonist binding occurring predominantly in the depolarized state. This proposal is in concert with the findings of Bean. Therefore, all dihydropyridine binding sites may represent identical gene products. Agonist binding produces allosteric effects, and at high levels of occupancy, there are alterations in the biophysical properties of the channel, with a net decrease in probability of channel opening. Our findings do not permit us to distinguish between these models for the intact heart cell system.

The biophysically derived model for calcium channel function of Kass predicts that ligands may bind to 2 different states of the calcium channel but that there is a unitary dihydropyridine binding site. He has found that BAY k 8644, under maximally negative holding potential, increases peak inward current and also increases the rate of channel inactivation. At less negative holding potentials, Kass has reported that BAY k 8644 decreases the inward current. This result was not due to use of a racemic mixture since the negative isomer BAY R 5417 had a dual action, depending on the holding potential. Our data are consistent with the view that dihydropyridines bind to different states of the channel with different binding parameters but that there is a unitary binding site. Thus, BAY k 8644, under some conditions, may promote channel inactivation. Our data demonstrate that high concentrations of BAY k 8644 decrease calcium influx and bind with low affinity to a site recognized by a calcium antagonist. It is possible that calcium channel activation is associated with formation of a state of the channel to which BAY k 8644 binds with low affinity and produces an antagonist effect.

Stereoispecificity appears to be an inherent property of the dihydropyridine binding site. Both verapamil and diltiazem have enantiomers of differing potency. Recent studies of the dihydropyridine 202-791 indicate that the (+) isomer acts as a calcium channel agonist while the (-) isomer acts as an antagonist. The stereoisomers of BAY k 8644 may have opposite effects as well. Thus, there is a fourth interpretation of our data that racemic BAY k 8644 may bind to the same site with two distinct affinities because it is composed of two different stereoisomers with opposite effects on the calcium channel. Arguing against the positive and negative effects being solely due to stereo specificity, however, is our finding that the pure levorotatory enantiomer, BAY R 5417, produced a positive inotropic effect at low concentration but that the positive inotropic effect was significantly attenuated at a higher con-
centration. Also, there is a preliminary report by Thomas et al. that the agonist action of the pure stereoisomer (−)-BAY k 8644 also decreases at high concentrations.

In summary, our results support the hypothesis that voltage-sensitive calcium channels have more than one binding site or state for dihydropyridines. The cultured ventricular cell experimental system facilitated demonstration that dihydropyridine ligands may bind to one or two sites of the channel; occupancy of one site augments calcium influx and contractility, while occupancy or promotion of transition to the other site diminishes calcium influx and contractility.

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