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Voltage-Dependent Binding of 1,4-Dihydropyridine Ca^{2+} Channel Antagonists and Activators in Cultured Neonatal Rat Ventricular Myocytes

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SUMMARY

Binding of 1,4-dihydropyridine Ca^{2+} channel ligands was characterized as a function of membrane potential using saturation, competition, and kinetic measurements in cultured neonatal rat ventricular myocytes. The 1,4-dihydropyridine antagonist [^{3}H]PN 200-110 bound to polarized cells (5.8 mM K^+) with a K_D value of 3.53×10^{-9} M and a B_{max} value of 50.1 fmol/mg of protein. In depolarized cells (50 mM K^+), a K_D value of 6.33×10^{-11} M was found, reflecting a 55-fold increase in affinity; B_{max} did not change upon depolarization. Dissociation rates (k_{-1}) of [^{3}H]PN 200-110 binding were faster in polarized cells (0.53 min^{-1}) than in depolarized cells (0.018 min^{-1}), but association rates (k_1 of 2.17×10^8 and 2.27×10^8 $min^{-1}M^{-1}$ were not different in polarized and depolarized cells. The K_D values calculated from the ratio of k_{-1}/k_1 accorded well with those determined from equilibrium binding assays. The enantiomers of Bay K 8644 and 202-791 and a series of nifedipine analogs inhibited specific binding of [^{3}H]PN 200-110 in depolarized cells. In polarized cells, the affinities of the *S*-enantiomers (activators) were close to those in depolarized cells; however, the affinities of *R*-enantiomers (antagonists) were 50- to 65-fold lower. The effects of both (*S*)- and (*R*)-Bay K 8644 on [^{3}H]PN 200-110 binding were mediated through increased apparent K_D values, without changes in B_{max} and n_H. In depolarized cells, *l*-D600 and *d*-D600 partially inhibited [^{3}H]PN 200-110 binding to a maximum of 71% and 56%, respectively; in polarized cells, *l*-D600 (*d*-D600 not measured) was ineffective on [^{3}H]PN 200-110 binding. *d*-(*cis*)-Diltiazem, but not *l*-(*cis*)-diltiazem, partially inhibited (maximum 30%) specific binding of [^{3}H]PN 200-110 in depolarized cells, but potentiated (maximum 79%) binding in polarized cells. The potentiating effect of *d*-(*cis*)-diltiazem was mediated through an increase in affinity without change in B_{max} of [^{3}H]PN 200-110 binding. (*S*)-Bay K 8644 potentiated $^{45}Ca^{2+}$ uptake into the cells, with an EC_{50} value of 4.26×10^{-10} M; concentrations higher than 10^{-7} M were inhibitory, producing a biphasic concentration-response relationship. (*R*)-Bay K 8644 inhibited 80 mM K^+-stimulated $^{45}Ca^{2+}$ uptake with an IC_{50} value of 2.11×10^{-9} M. These pharmacologic values correlate well with the binding affinities. Studies of [^{3}H]PN 200-110 binding and competition by enantiomers of Bay K 8644 and 202-791 in membrane preparations of the cells showed affinities similar to those in depolarized cells. It is concluded that membrane depolarization favors a state of voltage-sensitive Ca^{2+} channels that shows high affinity for the 1,4-dihydropyridine Ca^{2+} channel antagonists and that the affinities of the 1,4-dihydropyridine activators are relatively independent of membrane potential.

Several factors contribute to the observed patterns of selectivity of the Ca^{2+} channel antagonists, including the source of Ca^{2+} mobilized, the existence of subclasses of Ca^{2+} channels with different pharmacologic characteristics, and allosteric subtleties of interaction of drugs with channel binding sites. According to the modulated receptor hypothesis, originally advanced to explain the actions of local anesthetics on sodium channels (1, 2), drugs may selectively interact with specific binding sites in the resting, open, or inactivated channel states. Accordingly, the apparent affinity of a drug will vary according to its affinities for specific states and the time- and voltage-dependent equilibria between these states.

The frequency dependence of verapamil, D600, and diltiazem interactions was early recognized (3–6) and later studies have demonstrated that the actions of 1,4-dihydropyridine Ca^{2+} channel antagonists and activators, both quantitatively and qualitatively, are strongly dependent on membrane potential in a number of tissues, including cardiac muscle (7–11).

Radioligand binding to intact cells as a function of membrane potential represents another approach to the study of voltage-dependent interactions of 1,4-dihydropyridines with Ca^{2+} channels, including [^{3}H]nitrendipine and [^{3}H]PN 200-110, and indicates that binding is controlled by membrane potential. However, the results have not been consistent. Thus, an increase in

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ABBREVIATIONS: HEPES, 4-(2-hydroxyethyl)-1-piperezineethanesulfonic acid; TPP, tetraphenylphosphonium; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; CL, confidence limit.

the number of binding sites with little or no change in affinity was observed in isolated cardiomyocytes (12), cultured chick ventricular cells (13), skeletal muscle cells (14), and cardiac sarcolemmal vesicles (15) following depolarization. However, increased affinity without change in binding capacity has also been reported to occur with membrane depolarization (10, 16, 17). Schilling and Drewe (18), using a kinetic analysis of nitrendipine binding found a component of binding that was lost upon membrane hyperpolarization, consistent with an effect of membrane potential on affinity.

When cells or subcellular vesicles are used to study the effects of membrane potential on Ca^{2+} channel ligand binding, it is essential to have a preparation that is substantially homogeneous in terms of membrane potential. In enzymatically dispersed noncultured preparations, the presence of damaged cells, which are presumably depolarized, may contribute substantially to measured binding, particularly at low ligand concentrations. In the present study we used beating, intact cultured neonatal rat ventricular myocytes to determine 1,4-dihydropyridine Ca^{2+} channel ligand binding and competition in an antagonist/activator series as a function of membrane potential.

Materials and Methods

Cell culture. Ventricular cardiomyocytes were cultured by combining reported methods (19, 20) with modifications. Ventricles were removed from the hearts of 1- to 5-day neonatal rats and placed in an ice-cold Ca^{2+}- and Mg^{2+}-free salt solution (modified from Hanks' balanced salt solution) of the following composition (mM): NaCl, 127; KCl, 4.56; KH_2PO_4, 0.44; $NaHCO_3$, 4.16; Na_2HPO_4, 0.63; glucose, 5.56, and HEPES 20; pH 7.4. The ventricles were minced with scissors to fine particles and rinsed three or four times (20 ml of each) to remove blood. Dissociation of tissues into single cells was performed by repeated trypsinization. Tissues were incubated in 16 ml of 0.06% trypsin (Type II Crude; Sigma Chemical Company, St. Louis, MO), at 37° for 30 min in a trypsinizing flask with gentle stirring. After each 30-min period, the supernatant was collected and fresh trypsin solution was added. Tissues were usually completely digested after five cycles. Trypsin was prepared in the Ca^{2+}- and Mg^{2+}-free saline solution. The supernatant from the first trypsinization was discarded, and those from other cycles were mixed with an equal volume of culture medium containing 85% minimum essential medium (with Earle's salt), 15% horse serum, 25 mM HEPES, 4 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin-B. The mixture was centrifuged at 150 × *g* for 8 min, and the pellet was resuspended in culture medium and recentrifuged. The pellet from the second spin was suspended in an appropriate volume of culture medium to obtain a cell count and to determine viability, normally ≥95%, by Trypan blue exclusion. Cell density was adjusted to 5×10^5 cells/ml of medium. Cells were plated in Corning 35-mm plastic tissue culture dishes (2 ml per dish) for whole cell studies and in 100-mm dishes (15 ml per dish) for membrane fraction preparations. Culture dishes were maintained under an atmosphere of 5% CO_2 and 95% air at 37°. Confluent and spontaneously beating monolayers were fully developed 3 days after plating. Experiments were performed on cells after 5 to 7 days in culture.

Radioligand binding. Whole cell binding was performed with cells attached to the culture dishes. Medium was replaced with buffer containing varied concentrations of K^+. Cells were incubated with various concentrations of (+)-[^{3}H]PN 200-110 and unlabeled ligand at 37° for 90 min for equilibrium studies or for varying periods of time for kinetic studies. For saturation studies, 6 to 14 separate concentrations of radioligand were employed. At the end of the incubation period, the radioligand-containing buffer was aspirated under vacuum and the culture was washed rapidly, 3 times in 15 sec, with ice-cold buffer with the same concentration of KCl. Cells were extracted overnight with 1 ml of 0.5 N NaOH and radioactivity was determined by liquid scintillation counting. Nonspecific binding was routinely determined in the presence of 10^{-6} M unlabeled PN 200-110. Protein was determined by the method of Bradford (21). The resting buffer for binding contained (mM): NaCl, 127; KCl, 5.36; $CaCl_2$, 1.26; $MgCl_2$, 0.98; KH_2PO_4, 0.44; $NaHCO_3$, 4.16; Na_2HPO_4, 0.63; glucose, 5.56, and HEPES 20, pH 7.4. The K^+ concentration was varied by substituting NaCl with KCl on an equimolar basis.

Binding of [^{3}H]PN 200-110 was also studied with membrane preparations made from the cells grown in 100-mm culture dishes. Briefly, the medium was removed and the culture was rinsed with ice-cold Tris buffer (50 mM, pH 7.2). The cells were scraped from the plate and homogenized by six passes of a motor-driven (TRI-R-stirrer, setting 4) glass-Teflon pestle homogenizer (nominal clearance, 0.13–0.18 mm). The homogenate was centrifuged at 45,000 × *g* for 45 min and the pellet was suspended in ice-cold 50 mM Tris buffer for binding studies at a concentration of 150 to 200 μg of protein, determined by the method of Bradford (21), per 5-ml assay volume. The binding assay was essentially that established previously in our laboratory (22). Membrane protein was incubated with various concentrations of [^{3}H]PN 200-110 in 5 ml of Tris buffer (50 mM, pH 7.2) for 90 min at 25°. Nonspecific binding was routinely determined by incubation in the presence of 10^{-7} M unlabeled PN 200-110. Incubation was terminated by rapid filtration under vacuum through Whatman GF/B filters followed by 2 washes (total, 10 ml) with ice-cold Tris buffer using a cell harvester (Model M-24R; Brandel Instruments, Gaithersberg, MD). Radioactivity was determined by liquid scintillation counting. (+)-[^{3}H]PN 200-110 at a concentration of 8.52×10^{-11} M was used for competition binding studies.

The kinetics of [^{3}H]PN 200-110 binding were studied in depolarized and polarized cells. The time course of association was determined by incubating cells with various concentrations of [^{3}H]PN 200-110 for various periods of time. To determine the time course of dissociation, [^{3}H]PN 200-110 (9.47×10^{-11} M in depolarized cells and 2.84×10^{-10} M in polarized cells) was equilibrated with cells for 90 min. Dissociation was initiated by addition of unlabeled PN 200-110 (10^{-6} M) and bound [^{3}H]PN 200-110 was determined after various times. The dissociation of radioligand was described by the equation:

$$[RB_t] = [R_0]e^{-k_{-1}t} \quad (1)$$

$[RB_t]$ is the amount of radioligand bound at time t; $[R_0]$ is the amount of radioligand bound at time $t = 0$; k_{-1} is the dissociation rate constant. For association, a pseudo-first order process was assumed by holding the ligand concentration relatively constant by ensuring that less than 10% was bound. Thus, association was described by the equation:

$$[RB_t] = [R_e]\ (1 - e^{-k_{obs} \cdot t}) \quad (2)$$

$[Re]$ is the amount of radioligand bound at equilibrium; k_{obs} is the apparent association rate constant. The actual association rate constant K_1 was calculated from the slope of the plot of k_{obs} versus ligand concentration, $[L]$, or by the equation:

$$k_1 = (k_{obs} - k_{-1})/[L] \quad (3)$$

Membrane Potential Measurement. Membrane potential was estimated from [^{3}H]TPP distribution (23, 24). [^{3}H]TPP uptake was determined under the same conditions (temperature, buffer, and experimental procedures) as whole cell binding. The time course of [^{3}H]TPP uptake was determined by incubating cells with [^{3}H]TPP (0.06 μCi in 3-ml assay volume, 5.63×10^{-10} M) for varying periods of time. It was shown that the uptake reached a plateau at 90 min. Therefore, a 90-min incubation period was routinely used. Cells were presumed to be fully depolarized when the maximum K^+ concentration (150 mM) was employed; [^{3}H]TPP uptake under this condition was not related to membrane potential and was considered to be nonspecific. Thus, nonspecific uptake was determined in parallel and subtracted from the total value to obtain the actual uptake related to membrane potential.

The distribution of TPP is according to the Nernst equation and the transmembrane potential E can be estimated in the relationship:

$$E = (-RT/F)\ \ln\ ([TPP]_{in}/[TPP]_{out}) = -61\ \log\ ([TPP]_{in}/[TPP]_{out}) \quad (4)$$

Intracellular water volume was estimated using [^{14}C]inulin as an extracellular marker (25) and [^{3}H]H_2O to measure total volume. Cells 1 to 2×10^6/assay) were suspended in a total volume of 0.5 ml containing [^{14}C]inulin (0.5 μCi and [^{3}H]H_2O (2.0 μCi) at 4° and were immediately centrifuged for 1 min at 9000 × g. The supernatant was aspirated and an aliquot was counted by liquid scintillation to determine the extracellular concentration of [^{14}C]inulin. The total volume of the pellet was determined from the [^{3}H]H_2O content and the extracellular space was determined from the [^{14}C]inulin content of the pellet. Intracellular water volume was taken as the difference between total and extracellular spaces and is expressed per mg protein, assayed with the Bradford method.

$^{45}Ca^{2+}$ uptake. Ca^{2+} uptake studies were carried out at 37°. Cells were preincubated in a buffer without Ca^{2+} and with 0.2 mM EGTA for 5 min. This procedure reduces the exchange between $^{45}Ca^{2+}$ and membrane-bound $^{40}Ca^{2+}$ and permits the $^{45}Ca^{2+}$ uptake to be measured at short time intervals (26). Ca^{2+} uptake was initiated by replacing the preincubation buffer with 2 ml of resting or elevated-K^+ buffer containing Ca^{2+} (1 mM) with $^{45}Ca^{2+}$ (0.4–0.5 μCi/mol of Ca^{2+}). Uptake was allowed for 10 sec unless otherwise indicated and was terminated by rapidly removing the $^{45}Ca^{2+}$-containing buffer and washing the culture with ice-cold resting buffer three times within 15 sec. Cells were extracted overnight with 1 ml of 0.5 N NaOH and radioactivity was determined by liquid scintillation counting. Protein was assayed by the method of Lowry *et al.* (27). The resting buffer contained (mM): NaCl, 127; KCl, 4.56; $CaCl_2$, 1.0; $MgCl_2$, 0.98; $NaHCO_3$, 4.16; KH_2PO_4, 0.44; Na_2HPO_4, 0.63; glucose, 5.56, and HEPES, 20; pH 7.4. Equimolar substitution of NaCl with KCl was made when elevated K^+ concentrations were desired.

To observe the effects of a drug on $^{45}Ca^{2+}$ uptake, the drug was incubated with cells for 25 min in medium and then 5 min during the preincubation. $^{45}Ca^{2+}$ uptake was performed in the presence of the same concentration of the drug.

Materials. [^{3}H]PN 200-110 [isopropyl-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydro-5-methoxycarbonyl-2,6-dimethyl-3-pyridinecarboxylate; specific activity, 70.0 Ci/mmol], [^{3}H]TPP (specific activity, 35.5 Ci/mmol), [^{3}H]H_2O (specific activity, 1 mCi/ml), [^{14}C]inulin (specific activity, 3 mCi/g), and $^{45}Ca^{2+}$ (in the form of $CaCl_2$; specific activity, 23.8 mCi/mg) were purchased from DuPont-New England Nuclear (Boston, MA). Tissue culture medium, L-glutamine, serum, and antibiotics were obtained from GIBCO (Grand Island, NY). The enantiomers of Bay K 8644 [2,6-dimethyl-3-carbomethoxy-5-nitro-4-(2-trifluoromethylphenyl)-1,4-dihydropyridine] were the generous gift of Dr. A. Scriabine (Miles Institute for Preclinical Pharmacology, New Haven, CT) and the enantiomers of 202-791 [2,6-dimethyl-3-carbomethoxy-5-nitro-4-(2,1,3-benzoxadiazol-4-yl)-1,4-dihydropyridine] were the generous gift of Dr. P. R. Hof (Sandoz, Basel, Switzerland).

Statistics. Data were processed using an IBM personal computer. Equilibrium binding data were analyzed using the iterative curve fitting program BDATA (EMF Software, Knoxville, TN). Kinetic data were analyzed with the program KINETIC (Elsevier Software, New York, NY). Pharmacologic data and significance tests were analyzed using the pharmacologic programs of Tallarida and Murray (28). Results are presented with the standard error unless otherwise noted.

Results

[^{3}H]PN 200-110 binding to membrane preparations. Specific binding of [^{3}H]PN 200-110 was measured as a function of ligand concentration. It ranged from 80% of total binding at 4.6×10^{-11} M to 40% at the highest concentrations used in the study. These data support the presence of a single class of binding site (Hill coefficient, 1.04 ± 0.03), with a K_D value of 5.46 ± 0.32 × 10^{-11} M and a B_{max} value of 307 ± 12 fmol/mg of protein. When unlabeled nitrendipine (2×10^{-7} M) or nicardipine (2×10^{-7} M) was used to define the nonspecific binding, the analysis of specific [^{3}H]PN 200-110 binding generated very similar K_D, B_{max}, and n_H values. The inclusion of 80 mM KCl in the assay did not modify [^{3}H]PN 200-110 binding in this membrane preparation.

TABLE 1

Binding affinities of Bay K 8644 and 202-791 enantiomers in neonatal rat heart cell membranes

Ligand	IC_{50}[a]	K_I[a]	n_H
	M	M	
(S)-Bay K 8644	3.67×10^{-9} (1.31–10.3)	1.43×10^{-9} (0.51–4.02)	0.98
(R)-Bay K 8644	1.27×10^{-8} (0.47–3.44)	4.96×10^{-9} (1.84–13.4)	1.02
(S)-202-791	2.45×10^{-7} (0.86–6.96)	9.57×10^{-8} (3.34–27.1)	1.00
(R)-202-791	5.98×10^{-10} (1.33–26.9)	2.34×10^{-10} (0.52–10.5)	0.92

[a] Mean and 95% CL; n = 6.

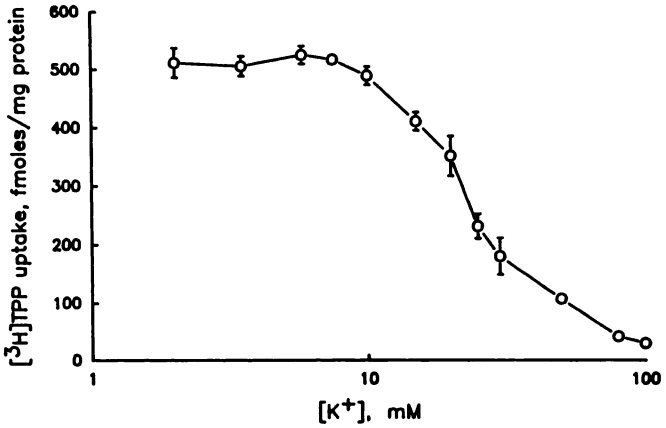

Fig. 1. [^{3}H]TPP uptake as a function of increasing K^+ concentration in neonatal rat heart cells. *Bars* indicate standard error of mean (n = 5).

The specific binding of [^{3}H]PN 200-110 to membrane preparations was inhibited in a concentration-dependent manner by (S)-Bay K 8644, (R)-Bay K 8644, (S)-202-791, and (R)-202-791. The K_I values and pseudo-Hill coefficients are summarized in Table 1. The inhibition curves are parallel to each other and the pseudo-Hill coefficients are close to unity, indicating apparently competitive interactions. The K_I values, which agree well with those obtained in smooth muscle and neurons[1] (29), were calculated by the procedure of Cheng and Prusoff (30). This procedure gives an accurate estimate of ligand affinity when the concentration of radioligand is close to its K_D value, as in the present study.

Calibration of membrane potential. [^{3}H]TPP uptake was measured in polarized (5.8 mM K^+) and depolarized (50 mM K^+) cells. Uptake reached a maximum between 60 and 90 min and was approximately 5 times greater in polarized cells. [^{3}H]TPP uptake decreased as the K^+ concentration increased to 100 mM (Fig. 1); the maximum [^{3}H]TPP uptake at 5.8 mM K^+ corresponds to 511 ± 17.9 fmol/mg of protein and did not change at K^+ concentrations below 7.5 mM. The EC_{50} value for K^+ inhibition of [^{3}H]TPP uptake was 23.35 mM (95% CL,

[1] X. Y. Wei *et al.*, submitted for publication.

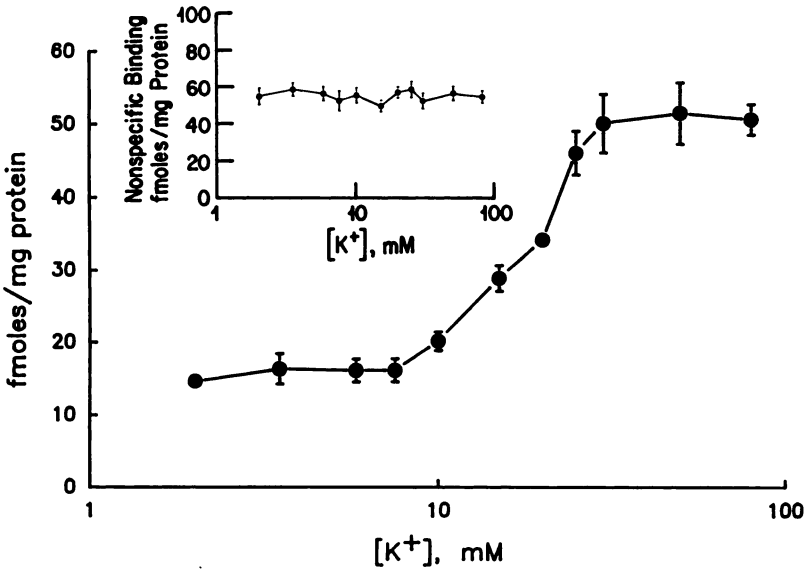

Fig. 2. Specific binding of [^{3}H]PN 200-110 (4.73×10^{-10} M) as a function of increasing K^+ concentration in neonatal rat heart cells. *Inset*, nonspecific binding of [^{3}H]PN 200-110 at different concentrations of K^+. *Bars* indicate standard error of mean ($n = 4$).

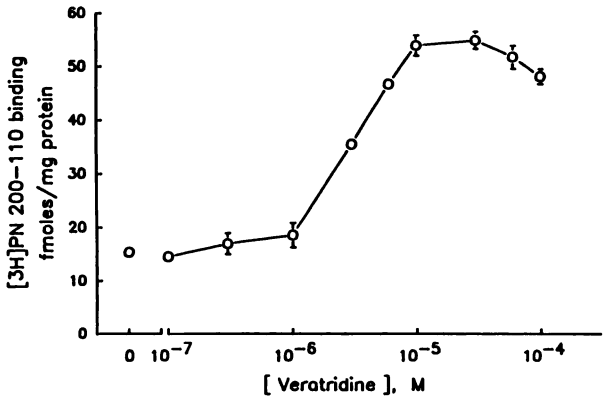

Fig. 3. Effect of veratridine on specific binding of [^{3}H]PN 200-110 binding in neonatal rat heart cells. Binding was measured at 4.73×10^{-10} M PN 200-110 and 5.8 mM K^+. *Bars* indicate standard error of mean ($n = 4$).

16.79–38.28 mM). The intracellular volume was determined to be 17.62 ± 0.37 μl/mg of protein or 3.68 ± 0.15 μl/10^6 cells. The membrane potential calculated according to Eq. 4 gave values of −103 mV and −57 mV at 5.8 mM and 50 mM K^+, respectively.

Characteristics of [^{3}H]PN 200-110 binding in intact cells. Binding of [^{3}H]PN 200-110 was measured as a function of different membrane potentials, achieved by varying K^+ concentrations in the buffer or by using the Na^+ channel-activator veratridine (31–33) in resting buffer. As shown in Fig. 2, specific binding of [^{3}H]PN 200-110 at a single concentration of 4.73×10^{-10} M increased, but nonspecific binding stayed constant at elevated K^+ concentrations. The change in binding occurred within a narrow range of K^+ concentrations of 7.5 mM to 30 mM, corresponding to a range of estimated membrane potential of 30 mV (Fig. 1). The EC_{50} value for K^+ enhancement of [^{3}H]PN 200-110 binding was 16.12 mM (95% CL, 11.22–23.14 mM), which was slightly, but not significantly, lower than the EC_{50} for depolarization.

To confirm that the effects of elevated K^+ on whole cell binding were due to membrane depolarization rather than to cation substitution, [^{3}H]PN 200-110 binding was measured in the presence of various concentrations of veratridine in the resting buffer. Fig. 3 shows that veratridine increased [^{3}H]PN 200-110 binding with an EC_{50} of 2.89 μM (95% CL, 1.06–7.85 μM). Binding of [^{3}H]PN 200-110 decreased when the veratridine concentration was higher than 3×10^{-5} M.

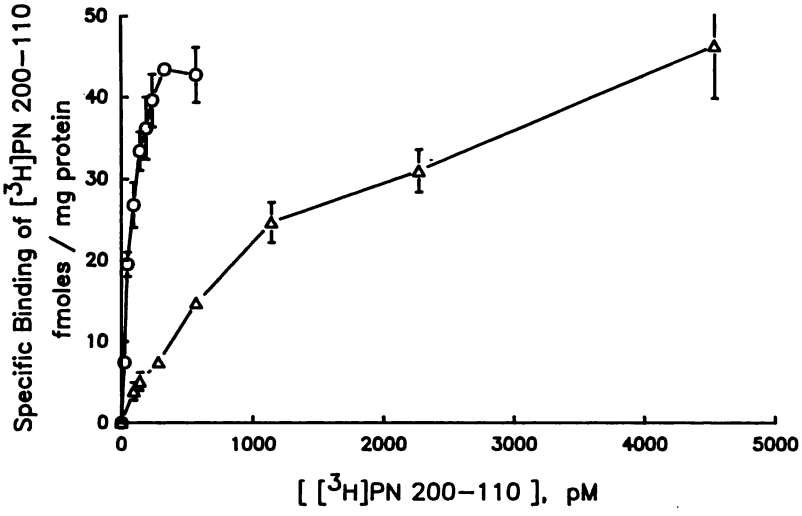

Fig. 4. Specific [^{3}H]PN 200-110 binding as a function of increasing concentration of [^{3}H]PN 200-110 in polarized cells (△) (5.8 mM K^+) and depolarized cells (○) (50 mM K^+). *Bars* indicate standard error of mean ($n = 5$ to 9).

TABLE 2

[^{3}H]PN 200-110 binding as a function of K^+ concentration in neonatal rat heart cells

Values are mean ± standard error.

K^+	K_D	B_{max}	n_H
mM	M	fmol/mg of protein	
80 (n = 7)	7.11 ± 0.85 × 10^{-11}	56.2 ± 3.6	1.04 ± 0.03
50 (n = 9)	6.33 ± 0.36 × 10^{-11}	47.2 ± 3.4	1.05 ± 0.05
30 (n = 4)	6.28 ± 0.61 × 10^{-11}	52.9 ± 1.8	1.01 ± 0.05
22 (n = 3)	1.92 ± 0.23 × 10^{-10}	52.4 ± 4.9	0.99 ± 0.04
15 (n = 7)	4.58 ± 0.97 × 10^{-10}	52.2 ± 5.2	1.00 ± 0.04
5.8 (n = 5)	3.53 ± 0.78 × 10^{-9}	50.1 ± 11.0	0.99 ± 0.02

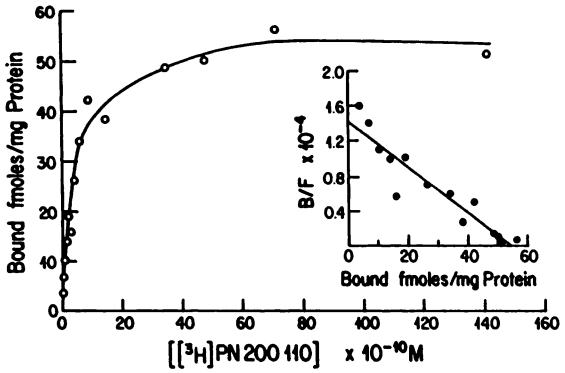

Fig. 5. Specific [^{3}H]PN 200-110 binding to neonatal rat heart cells in the presence of 20 mM K^+. *Inset*, Scatchard plot of specifically bound [^{3}H]PN 200-110. The K_D value for [^{3}H]PN 200-110 binding was 4.31 ± 0.38 × 10^{-10} M with a B_{max} of 60.5 ± 6.35 fmol/mg of protein. Depicted is one representative plot from five separate experiments.

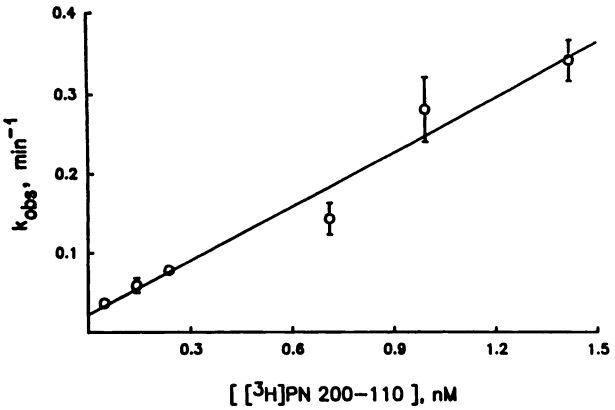

Fig. 6. The apparent association rate constant (k_{obs}) of [^{3}H]PN 200-110 as a function of radioligand concentration in depolarized cells (50 mM K^+). *Bars* indicate standard error of mean (n = 4).

Saturation binding of [^{3}H]PN 200-110 was measured at 5.8, 15, 22, 30, 50, and 80 mM K^+. Specific binding ranged from approximately 75% to 40% in 50 mM K^+ and from 30% to 10% in 5.8 mM K^+. Fig. 4 shows specific [^{3}H]PN 200-110 binding as a function of increasing concentration of the ligand at 5.8 and 50 mM K^+, respectively. These data show that, with increasing concentrations of K^+, the affinity of [^{3}H]PN 200-110 increased but that B_{max} remained constant (Table 2). At all K^+ concentrations, data were fit as a single binding site and Hill coefficients were close to unity. Comparison of the K_D values shows a 55.8-fold increase in affinity from resting (5.8 mM K^+) to depolarized (50 mM K^+) cells. In the presence of 20 mM K^+, binding of [^{3}H]PN 200-110 was measured using concentrations ranging from 2.23 × 10^{-11} M to 1.42 × 10^{-8} M (Fig. 5). Data

TABLE 3

Inhibition of [^{3}H]PN 200-110 binding in neonatal rat heart cells (depolarized)

Ligand	IC_{50}*	K_i*	n_H
	M	M	
(*S*)-Bay K 8644	1.16 × 10^{-9} (0.39–3.43)	4.92 × 10^{-10} (1.66–14.6)	1.10
(*R*)-Bay K 8644	3.07 × 10^{-9} (1.17–8.04)	1.31 × 10^{-9} (0.50–3.43)	1.09
(*S*)-202-791	8.73 × 10^{-8} (2.34–32.5)	3.72 × 10^{-8} (1.00–13.8)	0.96
(*R*)-202-791	3.84 × 10^{-10} (0.98–15.0)	1.63 × 10^{-10} (0.42–6.39)	0.93
Nitrendipine	2.81 × 10^{-10} (0.58–13.6)	1.20 × 10^{-10} (0.25–5.80)	0.85
Nifedipine			
3-NO_2	2.00 × 10^{-9} (0.49–8.25)	8.53 × 10^{-10} (2.07–35.1)	0.88
3-CN	7.32 × 10^{-9} (2.59–20.7)	3.12 × 10^{-9} (1.10–8.82)	1.07
—H	6.94 × 10^{-8} (1.86–25.8)	2.96 × 10^{-8} (0.79–10.9)	0.93
4-F	1.13 × 10^{-7} (0.33–3.84)	4.82 × 10^{-8} (1.42–16.4)	1.01
3-MeO	5.57 × 10^{-7} (1.36–22.8)	2.37 × 10^{-7} (0.58–9.72)	0.87
4-Cl	7.44 × 10^{-7} (2.10–26.4)	3.17 × 10^{-7} (0.90–11.2)	0.94
d-D600	4.74 × 10^{-7} (0.62–36.2)		0.53
l-D600	1.75 × 10^{-7} (0.31–10.0)		0.57

* Mean and 95% CL; n = 5 or 6.

TABLE 4

Inhibition of [^{3}H]PN 200-110 binding in neonatal rat heart cells (polarized)

Ligand	IC_{50}*	K_i*	n_H
	M	M	
(*S*)-Bay K 8644	3.48 × 10^{-9} (1.01–12.0)	3.00 × 10^{-9} (0.91–10.8)	0.91
(*R*)-Bay K 8644	9.86 × 10^{-8} (3.57–27.3)	8.49 × 10^{-8} (3.20–24.5)	1.08
(*S*)-202-791	3.23 × 10^{-7} (0.84–12.5)	2.78 × 10^{-7} (0.75–11.2)	0.93
(*R*)-202-791	9.93 × 10^{-9} (3.24–30.4)	8.55 × 10^{-9} (2.90–27.2)	0.89

* Mean and 95% CL; n = 5.

TABLE 5

Comparison of K_D and B_{max} values of [^{3}H]PN 200-110 in the presence of (*S*)- and (*R*)-Bay K 8644 in depolarized cells

Values are mean ± standard error; n = 4 or 5.

Ligand	K_D	B_{max}	n_H
	M	fmol/mg of protein	
Control	7.51 ± 0.60 × 10^{-11}	50.5 ± 0.5	0.98 ± 0.01
(*S*)-Bay K 8644			
10^{-9} M	1.83 ± 0.47 × 10^{-10}	54.5 ± 1.9	1.03 ± 0.02
10^{-7} M	5.27 ± 1.04 × 10^{-10}	51.7 ± 1.5	0.98 ± 0.01
Control	7.61 ± 0.08 × 10^{-11}	52.8 ± 2.0	0.99 ± 0.02
(*R*)-Bay K 8644			
10^{-9} M	1.44 ± 0.32 × 10^{-10}	54.5 ± 4.5	1.02 ± 0.03
10^{-8} M	2.89 ± 0.75 × 10^{-10}	51.4 ± 2.7	1.01 ± 0.01
10^{-7} M	3.96 ± 0.46 × 10^{-10}	53.1 ± 1.3	1.02 ± 0.01

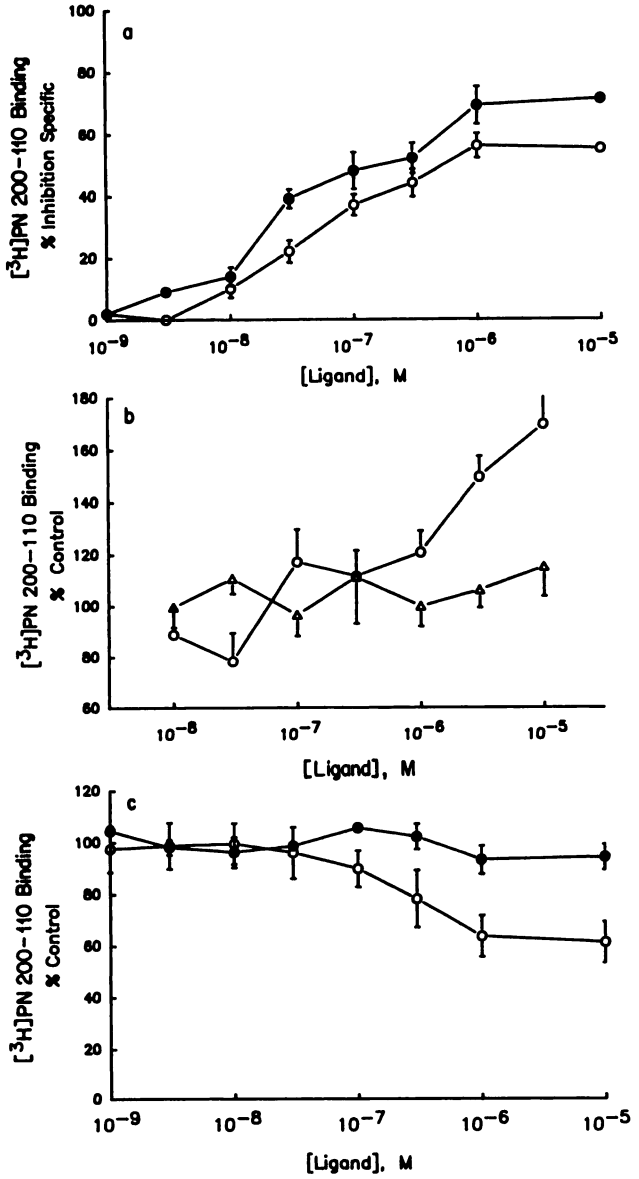

Fig. 7. a, Inhibition of specific binding of [^{3}H]PN 200-110 (8.52×10^{-11} M) in depolarized cells (50 mM K^+) by *l*-D600 (●) and *d*-D600 (○). *Vertical bars* represent standard error of mean ($n = 5$). b, Effects of *l*-D600 (△) and *d*-(*cis*)-diltiazem (○) on specific binding of [^{3}H]PN 200-110 (5.68×10^{-10} M) in polarized cells (5.8 mM K^+). *Vertical bars* represent standard error of mean ($n = 4$ or 5). c, Effects of *d*-(*cis*)-diltiazem (○) and *l*-(*cis*)-diltiazem (●) on specific binding of [^{3}H]PN 200-110 (8.52×10^{-11} M) in depolarized cells (50 mM K^+). *Vertical bars* represent standard error of mean ($n = 5$).

analysis (Fig. 5, *inset*) indicated a single binding site with a K_D value of $4.31 \pm 0.38 \times 10^{-10}$ M and a B_{max} value of 60.5 ± 6.35 fmol/mg of protein.

Kinetics of [^{3}H]PN 200-110 binding. Specific binding of [^{3}H]PN 200-110 was determined as a function of time in both depolarized cells (50 mM K^+) and polarized cells (5.8 mM K^+). In depolarized cells the time course of association was measured at various concentrations of [^{3}H]PN 200-110, ranging from below K_D to about 20 times higher than K_D. Association reached plateau values between 20 min at 1.42×10^{-9} M [^{3}H]PN 200-110 and 60 min at 4.73×10^{-11} M. The time course of association was monoexponential at all ligand concentrations studied; nonlinear least square analysis did not improve the data fit when

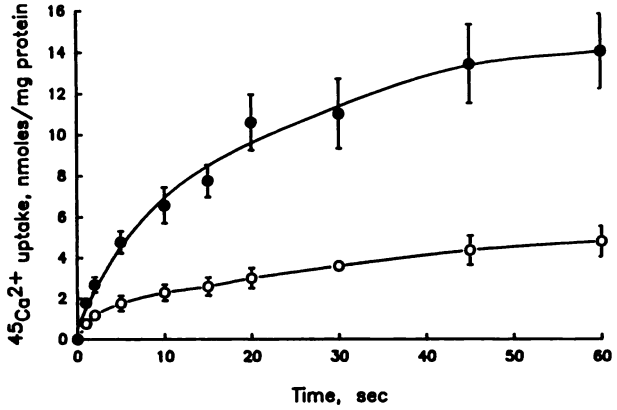

Fig. 8. Time course of $^{45}Ca^{2+}$ uptake into neonatal rat heart cells in 5 mM (○) and 80 mM (●) K^+. *Vertical bars* represent standard error of mean ($n = 7$).

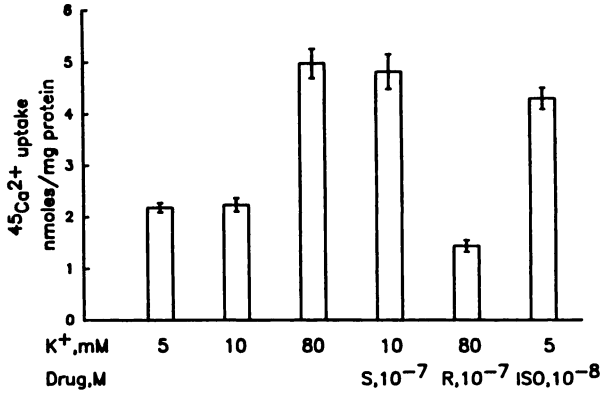

Fig. 9. $^{45}Ca^{2+}$ uptake at 10 sec into neonatal rat heart cells: stimulation by K^+ depolarization, (*S*)-Bay K 8644 (*S*), and isoproterenol (*ISO*) and inhibition by (*R*)-Bay K 8644 (*R*). *Bars* indicate standard error of mean ($n = 4$).

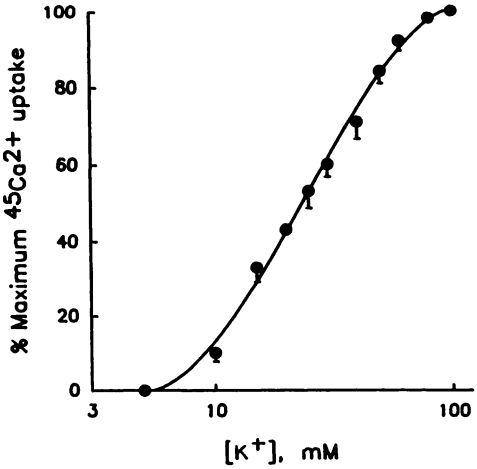

Fig. 10. Dose-response relationship for K^+-stimulated $^{45}Ca^{2+}$ uptake at 10 sec into neonatal rat heart cells. *Vertical bars* represent standard error of mean ($n = 4$). The EC_{50} for K^+ was 24.1 mM and maximum uptake was 5.1 ± 0.38 nmol/mg of protein.

two exponential processes were considered. The apparent rate constant, k_{obs}, versus radioligand concentration (Fig. 6) yielded k_1 as 2.27×10^8 $min^{-1} \cdot M^{-1}$. In polarized cells, the association rate of 2.17×10^8 min^{-1} M^{-1} for [^{3}H]PN 200-110 was monoexponential and not different from that in depolarized cells.

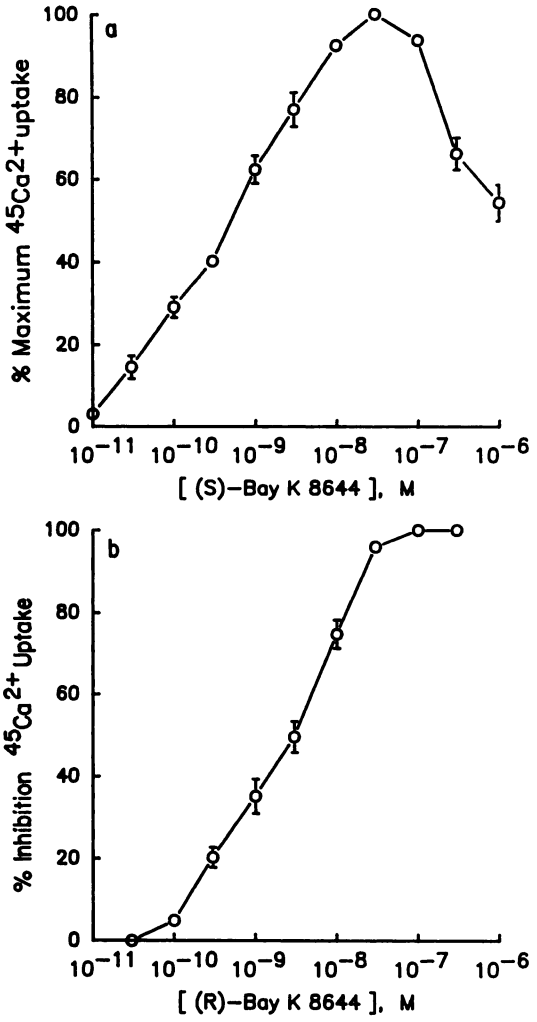

Fig. 11. Dose-response relationships of (*S*)-Bay K 8644 (a) and (*R*)-Bay K 8644 (b) as activator and antagonist, respectively, of $^{45}Ca^{2+}$ uptake into neonatal rat heart cells. Activation of $^{45}Ca^{2+}$ uptake was measured with 10 mM K^+ and antagonism with 80 mM K^+. *Vertical bars represent standard error of mean (n = 4).*

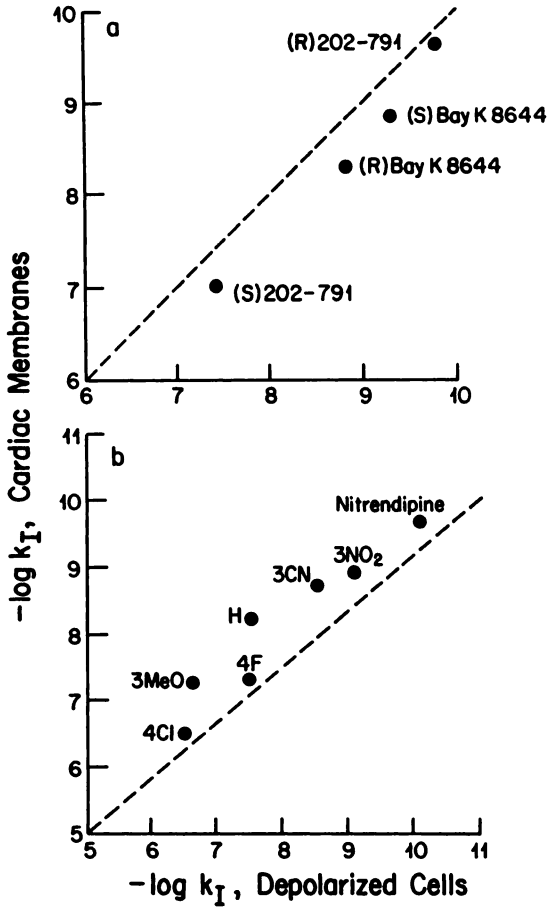

Fig. 12. a, Correlation between binding affinities in depolarized cells (50 mM K^+) and membrane preparations of neonatal rat heart cells for (*S*)-Bay K 8644, (*R*)-Bay K 8644, (*S*)-202-791, and (*R*)-202-791. The *dashed line* represents unit slope. b, Correlation between the binding affinities in depolarized cells (50 mM K^+) and membrane preparations of neonatal rat heart cells for a series of nifedipine derivatives (Table 3). The *dashed line* represents unit slope.

The dissociation time courses in both polarizd and depolarized cells were monoexponential. In depolarized cells, the dissociation rate constant k_{-1} was 0.018 ± 0.0012 min^{-1}. The ratio of k_{-1}/k_1 gave a K_D value of 7.92×10^{-11} M, which is similar to that, 6.33×10^{-11} M, determined from equilibrium binding. In polarized cells, however, the rate of dissociation was much faster, with a k_{-1} value of 0.53 ± 0.06 min^{-1}. The ratio of k_{-1}/k_1 was 2.44×10^{-9} M, which is also close to that, 3.53×10^{-9} M, determined from equilibrium binding. Thus, similar differences between the affinities of [^{3}H]PN 200-110 in depolarized and polarized cells were revealed from kinetic and equilibrium measurements of binding.

Inhibition of [^{3}H]PN 200-110 binding by 1,4-Dihydropyridine Ca^{2+} channel antagonists and activators. In depolarized cells (50 mM K^+), the enantiomers of Bay K 8644 and 202-791 and a series of nifedipine analogs inhibited the specific binding of [^{3}H]PN 200-110 (8.52×10^{-11} M) in a concentration-dependent and apparently competitive manner. K_I values and Hill coefficients are shown in Table 3. In polarized cells, the concentration-response curves of (*R*)-Bay K 8644 and (*R*)-202-791 were shifted to higher values by 50- to 65-fold (see Table 5). This shift is in good accord with that determined from [^{3}H]PN 200-110 saturation experiments. However, (*S*)-Bay K 8644 and (*S*)-202-791 inhibited binding of [^{3}H]PN 200-110 with K_I values at the two membrane potentials differing by only 6- to 7-fold (Tables 3 and 4). For both antagonist and activator enantiomers, the slope factors of the inhibition curves were not significantly different from unity under either depolarized or polarized conditions.

In depolarized cells, the effects of (*S*)- and (*R*)-Bay K 8644 on [^{3}H]PN 200-110 binding were determined by saturation analysis. Both (*S*)-Bay K 8644 and (*R*)-Bay K 8644 behaved as competitive antagonists of [^{3}H]PN 200-110 binding, increasing the apparent K_D value with no significant change in B_{max} values and Hill coefficients (Table 5).

Effects of D600 and diltiazem on [^{3}H]PN200 110 binding. In depolarized cells (50 mM K^+), the *l*- and *d*-isomers of D600 partially inhibited specific [^{3}H]PN 200-110 (8.52×10^{-11} M) binding (Fig. 7a). The *l*-isomer was slightly more effective than the *d*-isomer (Table 3) The maximum inhibition was 71% and 55% for the *l*- and *d*-isomers, respectively. The slope factors were significantly less than 1 (Table 3). In polarized (5.8 mM K^+) cells *l*-D600, at a concentration of 10^{-5} M, was virtually inactive against [^{3}H]PN 200-110 (5.68×10^{-10} M) binding (Fig. 7b).

d-(*cis*)-Diltiazem at high concentrations (above 10^{-6} M) in-

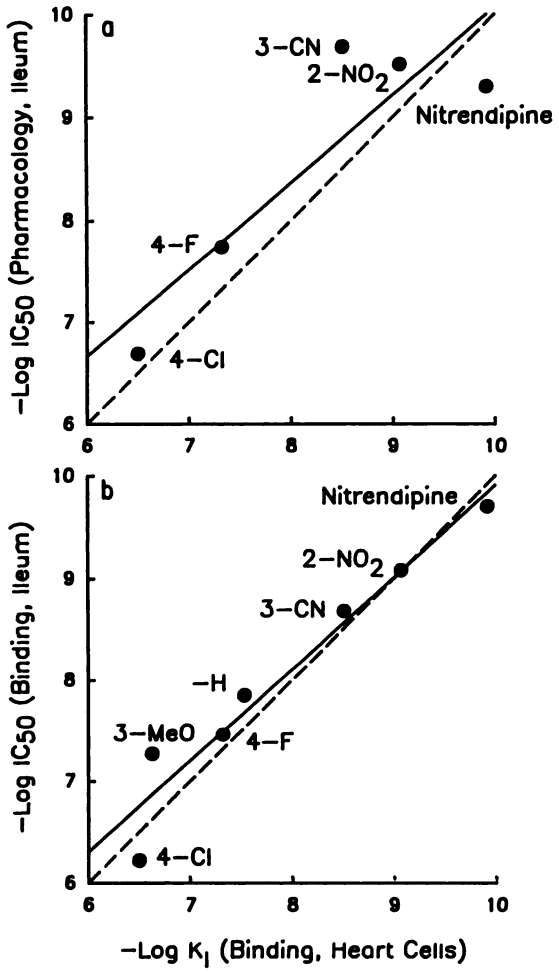

Fig. 13. a, Correlation between binding affinities in depolarized neonatal rat heart cells (50 mM K^+) and pharmacologic activities (antagonism of K^+ depolarization-induced tension responses) in guinea pig ileal smooth muscle preparations (22) for a series of nifedipine analogs. The *solid line* represents linear regression and the *dashed line* unit slope. b, Correlation between radioligand binding affinities in depolarized neonatal rat heart cells (50 mM K^+) and in microsomal membranes from guinea pig ileal smooth muscle preparations (22) for a series of nifedipine analogs. The *solid line* represents linear regression and the *dashed line* unit slope. The binding data for the ileal smooth muscle were measured using [^{3}H] nitrendipine competition under conditions very similar to those employed for the cardiac membranes.

hibited a small fraction only (maximum 39%) of [^{3}H]PN 200-110 binding in depolarized cells (Fig. 7c). *l*-(*cis*)-Diltiazem was ineffective. However, in polarized cells, *d*-(*cis*)-diltiazem significantly potentiated [^{3}H]PN 200-110 binding (Fig. 7b); at 10^{-5} M *d*-(*cis*)-diltiazem binding of PN 200-110 was increased by 70%. The effect of diltiazem was to increase the affinity without change of B_{max} of [^{3}H]PN 200-110 binding. K_D values were 3.53 ± 0.78 × 10^{-9} M and 6.9 ± 0.76 × 10^{-10} M and B_{max} values were 50.1 ± 11 and 57.2 ± 9.8 fmol/mg of protein, respectively, as control and in the presence of diltiazem (10^{-5} M).

$^{45}Ca^{2+}$ uptake. The time course of $^{45}Ca^{2+}$ uptake into neonatal rat heart cells is shown in Fig. 8. The uptake reached a maximum between 45 and 60 sec. At 10 sec, the uptake was 2.29 ± 0.40 and 6.56 ± 0.87 nmol/mg of protein in 5 mM and 80 mM K^+, respectively, approximately 50% of the maximum uptake at 60 sec. Ca^{2+} uptake was routinely measured at 10 sec in other assays.

Ca^{2+} uptake was sensitive to K^+ depolarization, 1,4-dihydropyridines, and a β-adrenergic agonist (Fig. 9). K^+ (80 mM), (*S*)-Bay K 8644 (10^{-7} M), and isoproterenol (10^{-8} M with 1-min preincubation with cells) stimulated Ca^{2+} uptake to similar levels. (*R*)-Bay K 8644 (10^{-7} M) inhibited 80 mM K^+-stimulated Ca^{2+} uptake to a level that was below the control value at 5 mM K^+. A K^+ concentration of 10 mM was optimal for stimulation of uptake by (*S*)-Bay K 8644.

The K^+ concentration-response relationship for stimulated Ca^{2+} uptake (Fig. 10) yielded an EC_{50} value of K^+ of 24.1 mM (95% CL, 15.7–36.9 mM). (*S*)-Bay K 8644 stimulated Ca^{2+} uptake at concentrations lower than 3 × 10^{-8} M, with an EC_{50} value of 4.26 × 10^{-10} M (Fig. 11a). At concentrations higher than 10^{-7} M, (*S*)-Bay K 8644 inhibited Ca^{2+} uptake, thus producing a biphasic dose-response curve. At 10^{-6} M (*S*)-Bay K 8644 Ca^{2+} uptake was 51% of the maximally stimulated level at 3 × 10^{-8} M. (*R*)-Bay K 8644 inhibited dose-dependently Ca^{2+} uptake stimulated by K^+ (80 mM) depolarization, with an IC_{50} value of 2.11 × 10^{-9} M (Fig. 11b).

The pharmacologic activities of (*S*)- and (*R*)-Bay K 8644 were in good agreement with binding affinities. However, when $^{45}Ca^{2+}$ uptake was measured without preincubating the cells in the Ca^{2+}- and Mg^{2+}-free buffer, the observed uptake was much lower (1.09 ± 0.08 nmol/mg of protein) and was sensitive to neither depolarization nor 1,4-dihydropyridines.

Discussion

Analysis of voltage-dependent interactions of Ca^{2+} channels with specific ligands is of fundamental importance to an understanding of the mechanisms of channel modulation. In this study, we demonstrate that binding of 1,4-dihydropyridine Ca^{2+} channel antagonists to Ca^{2+} channels in cultured rat ventricular cells is voltage dependent. Binding increases as a function of membrane depolarization, achieved either by elevation of extracellular K^+ concentration or by the Na^+ channel activator veratridine. Binding at K^+ concentrations below 30 mM is essentially a linear function of membrane potential and concentrations of K^+ above 30 mM are not associated with any further increase in binding. Concentrations of veratridine above 3 × 10^{-5} M inhibit binding, in accord with the direct inhibition of nitrendipine binding previously described in guinea pig ileal membranes (22) and the blockade of Ca^{2+} currents in neurons (34). That veratridine and other ligands, including local anesthetics, interact at both Na^+ and Ca^{2+} channels is consistent with structural homology between these channels (35, 36).

Measurement of membrane potential from [^{3}H]TPP distribution is an indirect procedure and may not yield the absolute values of membrane potential. The limitations include distribution of the lipophilic cation across intracellular membranes (24), difficulty in estimating extracellular space in cultured cells, and the use of cell suspensions for estimation of cell volume. If a constant intracellular K^+ concentration of 140 mM is assumed for this cardiac cell preparation, membrane potentials of −84 mV and −27 mV are predicted by the Nernst equation at 5.8 and 50 mM K^+, respectively. These predicted values are significantly different from those calculated from [^{3}H]TPP distribution. However, the [^{3}H]TPP distribution data are of use in demonstrating that K^+ concentrations do produce changes in membrane potential.

[^{3}H]PN 200-110 binds to a single class of binding sites at all membrane potentials. The density of this site stays constant,

but its affinity varies according to membrane potential. The K_D value of [^{3}H]PN 200-110 in depolarized cells (6.28×10^{-11} M to 7.11×10^{-11} M) is very similar to that in the membrane preparations (5.46×10^{-11} M) and is in good agreement with values from other membrane systems (37–40). Competition studies of the inhibition of specific [^{3}H]PN 200-110 binding by the enantiomers of Bay K 8644 and 202-791 reveal a very close agreement, virtually 1:1, between affinities measured in depolarized cells and in membrane preparations from cardiac tissue (Fig. 12a). This same relationship extends to the larger series of 1,4-dihydropyridine analogs of nifedipine (Fig. 12b). Additionally, the same structure-activity relationships are observed for 1,4-dihydropyridine binding to depolarized cardiac cells and inhibition of K^+ depolarization-induced tension responses in ileal smooth muscle (Fig. 13a) and for binding to depolarized cardiac cells and membrane preparations from ileal smooth muscle (Fig. 13b) (22). These correlations demonstrate that depolarized cardiac cells behave very similarly to membrane fragments in the binding of 1,4-dihydropyridines. Thus, a similar state of the channel recognized by 1,4-dihydropyridines may be formed on reversible depolarization or in membrane preparations.

The affinity of [^{3}H]PN 200-110 is much lower, however, in polarized cells, with an approximately 55-fold increase in K_D value relative to depolarized cells. Competition binding studies with the antagonist enantiomers yield similar shifts in affinity. The K_I values of (*R*)-Bay K 8644 and (*R*)-202-791 as inhibitors of [^{3}H]PN 200-110 binding are increased by 50- to 65-fold. The affinities of 1,4-dihydropyridine Ca^{2+} channel antagonists clearly increase with increasing membrane depolarization. Similar observations have been made previously (10, 16, 17). Electrophysiologic evidence suggests that, in cardiac tissues, membrane depolarization favors an inactivated channel state (7, 9, 11), to which Ca^{2+} channel antagonists bind with high affinity. Binding affinities of 1,4-dihydropyridine antagonists to the resting states of cardiac Ca^{2+} channels are lower. Thus, the measured affinity of a 1,4-dihydropyridine will be determined by its affinities for the several channel states and by the fractional availability of each channel state.

The effect of depolarization on 1,4-dihydropyridine affinity is exerted only on the dissociation rate, which is some 30-fold less in depolarized cells. The association rate is rapid and independent of membrane potential. Thus, depolarization does not increase the access of the 1,4-dihydropyridine to the binding site but rather decreases the departure rate. This would provide an argument against the "guarded receptor" hypothesis (41), according to which membrane potential modulates ligand affinity by alteration of the amount of time during which the receptor binding site is available to the ligand. However, if 1,4-dihydropyridines associate with the binding sites by diffusion through the lipid bilayer (42), then the effects of membrane potential on the true association rate may be obscured.

Although the affinity differences measured in the binding studies are significant, they are smaller than the 100- to 1000-fold differences measured electrophysiologically. Such discrepancies are likely due to the difficulty in obtaining a fully polarized condition in the spontaneously beating heart cells in culture, which are presumably partially depolarized even at the lowest concentrations of K^+ (5.8 mM) employed, whereas voltage can be clamped precisely by electrophysiologic techniques. Additionally, the 1,4-dihydropyridines themselves and elevated K^+ (80 mM) reduce the frequency of spontaneous contractions and responses to modest elevations of K^+. Thus, our experimental conditions do not provide precise clamping of membrane potential.

Weiland and Oswald (43) reported in rat brain membranes a biphasic association but a monophasic dissociation process of [^{3}H]PN 200-110 binding; the rate of the fast component of association was dependent on the ligand concentration. In the same study, they found that both association and dissociation of [^{3}H]nitrendipine were biphasic. The results were interpreted as reflecting binding states of Ca^{2+} channel with different affinities. In our study, at all concentrations of radioligand, there are single rate constants of association and dissociation in both polarized and depolarized states. These results are consistent with a single affinity state in both polarized and depolarized cells, with the faster rate of dissociation determining the lower affinity in polarized cells. The discrepancy between our results and those of Weiland and Oswald (43) may be due to different tissues (heart versus brain), temperatures (37° versus 20°) or preparations (whole cell versus membrane).

Binding studies with membrane preparations generally reveal a single high affinity site for 1,4-dihydropyridines, consistent with our results with whole cells. However, the presence of two binding sites in some preparations has also been reported (44–47). The relationship of the low affinity sites identified in membrane preparations to those found in polarized cells as reported in this study remains to be determined.

Studies of the effects of depolarization on 1,4-dihydropyridine binding in cells have yielded different conclusions. Changes in numbers of binding site rather than affinity have been seen in some preparations (12–15). As noted in the introduction, it is likely that such results arise from the use of preparations with mixed populations of membrane potential. Cells or vesicles that are damaged and, thus, depolarized before and during the binding assay will contribute, at least in part, to the observed high affinity binding under nondepolarizing conditions (15). The observed increase in receptor density upon depolarization then reflects the transition from an unmeasured low affinity state to the already detected high affinity state. This limitation presumably does not apply to the study by Lee *et al.* (13), which used spontaneously beating cultured chick ventricular cells. However, this study measured binding indirectly by displacement, because the levels of specific binding were too low to permit construction of complete saturation curves under both polarized and depolarized conditions. Additionally, PN 200-110 showed significantly lower affinity, 10^{-9} M, in this preparation than reported in other systems.

That the Ca^{2+} channel activators (*S*)-Bay K 8644 and (*S*)-202-791 showed very little difference (6- to 7-fold) in affinities in polarized and depolarized cells contrasts with the 50- to 65-fold changes measured for the antagonist 1,4-dihydropyridines. Under both potential conditions, the high binding affinities of the activators are similar to those in membrane preparations of the same cells and smooth muscle (29). If we accept the proposal from electrophysiologic studies that Ca^{2+} channels in cardiac tissues are predominantly in the resting state under polarized conditions and in the open or inactivated state under depolarized conditions, then our results suggest that activators bind with similar high affinities regardless of channel state, whereas antagonists bind with high affinity to the inactivated and/or open states and with significantly lower affinities to

resting states of the channel. However, the small differences in affinity observed with activators likely reflect a slightly more favorable binding to the open state of the channel, which is formed upon depolarization and stabilized by activators. Our results agree with those of Williams *et al.* (48), who have reported that (*S*)-202-791 activates cardiac Ca^{2+} current independent of membrane potential and who have suggested that the affinities of activators are independent of membrane potential. A similar observation was made by McCarthy and Cohen (49). Other electrophysiologic studies with Bay K 8644 or its activator enantiomer have shown that activators potentiate or inhibit Ca^{2+} current at polarized and depolarized potentials, respectively (8, 11, 50, 51). Thus, our binding results and the reported electrophysiologic data together indicate that Ca^{2+} channel activators bind to the Ca^{2+} channel with high affinity with little influence of membrane potential. Accordingly, activators may interact with either open or inactivated channel states to promote or block function, respectively, depending on the dominant channel population. It would, thus, be predicted that activators will not serve as antagonists in preparations in which the Ca^{2+} channels do not adopt an inactivated state. This has been observed in rat anterior pituitary cells (49). Additionally, direct binding of (*S*)-[^{3}H]Bay K 8644 to intact cells should be independent of membrane potential. This has been confirmed.[2]

Discrepancies between binding and pharmacologic activities of 1,4-dihydropyridine and other Ca^{2+} channel ligands are of interest because they raise critical questions concerning the relationship between binding sites and functional Ca^{2+} channels. In cardiac tissues it has been shown that pharmacologic activities of Ca^{2+} antagonists, but not activators, are generally 100- to 1000-fold less than their binding affinities (52–55). Although, in principle, a number of factors may be responsible for the discrepancies (56), state-dependent interactions of Ca^{2+} channels with antagonists may be the most plausible. Electrophysiologic studies predict that affinity for the inactivated state is some 1000-fold higher than for the resting state (6, 7, 57). The high affinity binding in depolarized cells and membranes reflects interaction of ligands with inactivated channels (9, 58) and the low pharmacologic activity of antagonists represents interaction with low affinity states of the channels, as measured in the polarized cells in this study and by Kokubun *et al.* (10).

In this study we have observed, in agreement with previous reports (59, 60), that (*S*)-Bay K 8644 stimulated Ca^{2+} influx into heart cells with a potency similar to the binding affinity. Furthermore, (*R*)-Bay K 8644 inhibited K^+ (80 mM)-stimulated Ca^{2+} influx at concentrations that inhibited binding in membranes and depolarized cells. Because the effect of the antagonist on Ca^{2+} influx was measured under depolarized conditions and Ca^{2+} channels do inactivate in cardiac tissue (11), it is plausible that the high affinity functional effect of (*R*)-Bay K 8644 is due to its binding to inactivated channels. The Ca^{2+} influx results thus provide further support to the model of state-dependent interactions. These data contrast with the situation in chick neural retina cells, where inhibition of Ca^{2+} uptake by antagonists occurs at 100-fold higher concentrations than does inhibition of binding. Ca^{2+} channels in this neuronal preparation appear to inactivate very slowly and the low affinity pharmacology of the 1,4-dihydropyridine antagonists may reflect lack of access to a high affinity inactivated state.[1]

A biphasic effect of (*S*)-Bay K 8644 on Ca^{2+} influx has been observed in this study. (*S*)-Bay K 8644 at concentrations below 3×10^{-8} M potentiated Ca^{2+} influx, but reduction of potentiation occurred at higher concentrations. Similar observations have been made with racemic Bay K 8644 on Ca^{2+} influx into cardiac cells (13, 59). In smooth muscle tissues, we have shown that the dual effects are not due to the involvement of the antagonist isomer (29). The biphasic responses to Ca^{2+} channel activators are likely explained by either the presence of distinct activator and antagonist binding sites (10, 61, 62) or by the state-dependent expression of functional activity of activator binding, as discussed previously.

The effects of phenylalkylamine compounds on 1,4-dihydropyridine binding to Ca^{2+} channels have been characterized in many investigations (63). Verapamil and D600 generally act as partial inhibitors of 1,4-dihydropyridine binding in various membrane preparations (23, 64, 65). In depolarized cells, we have shown a similar partial inhibition of [^{3}H]PN 200-110 binding. Furthermore, we found that the interaction between D600 and 1,4-dihydropyridines is voltage dependent. D600 is essentially ineffective against [^{3}H]PN 200-110 binding in polarized cells. Similarly, the enantiomers of desmethoxyverapamil do not affect [^{3}H]PN 200-110 binding to polarized cardiac cells (66). These observations suggest that the allosteric linkage between the phenylalkylamine and 1,4-dihydropyridine binding sites is weaker under polarized than under depolarized conditions. Electrophysiologic data have shown that blockade of Ca^{2+} current by verapamil and D600 is voltage dependent; these agents are more potent antagonists at depolarized potentials (3, 4, 67, 68). The approximately 3-fold stereoselectivity of *l*-D600 over *d*-D600 in depolarized cells is, however, significantly smaller than the reported values in smooth muscle and cardiac tissues (22, 69, 70).

The reported effects of diltiazem on 1,4-dihydropyridine binding to membrane preparations are quite variable. Diltiazem has been shown to potentiate (*inter alia*, Refs. 22, 46, 65, and 71) to inhibit (72, 73), or to be without effect on (17, 52) 1,4-dihydropyridine binding. Such variable effects are dependent on experimental conditions including the concentration of diltiazem and the temperature (22, 74). At 37°, however, potentiation of 1,4-dihydropyridine binding is usually observed (63). With isolated cardiac myocytes, Green *et al.* (12) reported inhibition of nitrendipine binding by diltiazem under depolarizing conditions but slight potentiation under resting conditions. Our results on intact and functional heart cells indicate that the effects of diltiazem on PN 200-110 binding are membrane potential dependent, in accord with electrophysiologic studies (5, 6). Diltiazem enhanced PN 200-110 binding in polarized cells, by increasing affinity without change in binding site density, but inhibited a small fraction of binding in depolarized cells. Similar observations have been made by Porzig and Becker (66). The absence of diltiazem potentiation of [^{3}H]PN 200-110 binding in depolarized cells contrasts with the data from membrane preparations. However, the ineffectiveness of *l*-(*cis*)-diltiazem in whole cell binding accords both with its absence of effect in membrane preparations and its low pharmacologic potency (71, 75). That the interactions of D600 and diltiazem with [^{3}H]PN 200-110 binding in depolarized cells differ from those reported for membrane fractions suggests the

[2] J. Ferrante, A. Rutledge, E. Luchowski, and D. J. Triggle, submitted for publication.

availability of channel states in the membrane preparation that are not due solely to depolarization. Thus, despite the apparent close similarity of 1,4-dihydropyridine binding in depolarized cells and membranes, differences in allosteric interactions between binding sites may exist.

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