0026-895X/87/030221-11$02.00/0

MOLECULAR PHARMACOLOGY, 31:221-231

A Comparison Between the Binding and Electrophysiological Effects of Dihydropyridines on Cardiac Membranes

SUSAN L. HAMILTON, ATSUKO YATANI, KEVIN BRUSH, ARNOLD SCHWARTZ, and ARTHUR M. BROWN

Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, Texas 77030 (S.L.H., A.Y., K.B., A.M.B.) and Departments of Pharmacology and Cell Biophysics, Physiology and Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267 (A.S.)

Received October 6, 1986; Accepted December 23, 1986

SUMMARY

To investigate the mechanism of action of dihydropyridines on calcium channels, both receptor binding and the electrophysiological effects of optically pure enantiomers of Bay k 8644, Sandoz 202-791, nimodipine, and PN200-110 were studied in a guinea pig ventricular preparation. The radioligand binding studies are consistent with other reports that the inhibitory and excitatory dihydropyridines share a single binding site. The patch clamp method was used for recording whole cell calcium currents. (−)-Bay k 8644 and (+)-202-791 produced voltage-dependent increases in the calcium currents. The magnitude of the effect was dependent on the holding potential. At holding potentials between −40 and −90 mV these activators increased calcium currents in a concentration-dependent manner with EC_{50} values of 25 nM and 80 nM, respectively. The inhibitors (+)-Bay k 8644, (−)-202-791, (+)- and (−)-nimodipine, and (+)-PN200-110 blocked the calcium currents with potencies that depended upon holding potential. The IC_{50} values for these enantiomers measured at a holding potential of −80 mV were, respectively, 8000, 200, 2000, 450, and 400 nM, and IC_{50} values measured at a holding potential of −30 mV were 26, 1.0, 52, 4.0, and 4.5 nM. The dissociation constants calculated for some dihydropyridines are similar to the K_d values determined by radioligand binding. However, for other dihydropyridines, large discrepancies between the concentrations giving rise to half-maximal electrophysiological effects and the K_d values from binding studies could not be reconciled by voltage-dependent binding alone. We suggest that each dihydropyridine also produces unique effects on the voltage-dependent gating of calcium channels.

Calcium channels are widely distributed membrane proteins which are important for electrical excitability, excitation-contraction coupling, excitation-secretion coupling, and other cellular functions (1, 2). A class of organic compounds known as DHPs functions either to block or activate voltage-dependent calcium channels in a variety of tissues and is, therefore, of considerable interest for both therapeutic and experimental purposes. Determination of the affinities of the DHPs from radioligand binding studies using cardiac membrane fragments has demonstrated a significant difference between these values and the affinities estimated from electrophysiological concentration-response data with intact myocytes. The concentrations of these drugs which give rise to a half-maximal response electrophysiologically are several orders of magnitude greater than the K_d values determined from radioligand binding. Recent electrophysiological experiments have demonstrated a voltage dependence of the DHP antagonist action and this has been postulated to account for the differences between the binding affinity and the affinity determined from the pharmacological effects of these drugs (3, 4). However, the experimental situation is complicated since it has been shown that the Ca channel antagonist nitrendipine as well as the agonist Bay k 8644 have dual stimulatory and inhibitory effects on Ca currents, I_{Ca} (5, 6), as well as on contraction in both heart and vascular smooth muscle (7–10). As these compounds are racemic mixtures, the biphasic effects could be due to the oppositely acting isomers. The enantiomers of the DHPs Sandoz 202-791 (11, 12) and Bay k 8644 (13) have been shown to have opposing actions on calcium currents in intact cells. Differences in the activities of the enantiomers of PN200-110 have also been reported (14).

Our first study (12) provided evidence that the optically pure DHP enantiomers, (+)- and (−)-202-791, produced opposite effects as determined in electrophysiological and pharmacological experiments and that a complex voltage dependency was involved, primarily in the action of the inhibitory enantiomer.

This work is supported by Grant DHHS IROI HL32935 from the Department of Health and Human Services and a grant from the American Heart Association, Texas Affiliate, to S. L. H., Grants DHHS 2ROI HL25145-06, DHHS IROI HL33662-01, and DHHS 5ROI-NS11453-11 from the Department of Health and Human Services and a grant from Miles Laboratories to A. M. B.; Grant ROIHL 22619-06 from the Department of Health and Human Services to A. S.; and Grant 851159 from the American Heart Association to A. Y.

ABBREVIATIONS: EDTA, ethylenediaminetetraacetate; MOPS, 4-morpholinepropanesulfonic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′N′*-tetraacetic acid; I-V, current-voltage.

To examine these phenomena further, we have studied the effects of optically pure enantiomers, including Bay k 8644, nimodipine, and PN200-110, on the Ca currents recorded under voltage clamp conditions in enzymatically isolated guinea pig myocytes. Additional experiments with the enantiomers of Sandoz 202-791 were also performed. For comparison, we have characterized the effects of these compounds on the binding of radiolabeled DHPs to membrane fragments derived from guinea pig ventricular tissue.

Experimental Procedures

Materials. [^{3}H]-(±)-Nitrendipine (78 Ci/mmol) was purchased from New England Nuclear. [^{3}H]-(+)-PN200-110 (82 Ci/mmole) was purchased from Amersham. Unlabeled nitrendipine, Bay k 8644, nimodipine and purified nimodipine enantiomers were supplied by Miles Laboratory. (+)-PN200-110 and enantiomers of 202-791 were obtained from Sandoz Laboratories. These enantiomers are all at least 95% pure.

Methods. Membranes were prepared from guinea pig ventricular tissue as follows. Ventricular tissue was removed from 10 albino guinea pigs and placed in 20 ml of ice-cold buffer I [25 mM KCl, 40 mM sodium tetraborate hydrochloride (pH 6.8), 5 mM EDTA] for 1 hr. The tissue was removed and minced with scissors. Buffer II [10 mM MOPS (pH 7.4), 10% sucrose, 100 μM aminobenzamidine, 1 μg/ml pepstatin, 5 μg/ml leupeptin, 5 μg/ml aprotinin, 10 μM phenylmethylsulfonyl fluoride] was added to give 10 ml of buffer per g of tissue, and the tissue was homogenized three times for 15 sec each at high speed in a commercial Waring blender. The homogenate was centrifuged for 10 min at 4,500 ×*g* in a JA 20 rotor in a Beckman J2-21 centrifuge. The supernatant (S1) was removed and centrifuged at 90,000 × *g* for 30 min in a 60 Ti rotor in an ultracentrifuge to give a pellet (P2) and a supernatant (S2). The pellet from the second centrifugation (P2) was resuspended in 6 ml of 10 mM MOPS, containing protease inhibitors, at the concentrations described above. This fraction was used in the binding experiments described in Figs. 1–6.

[^{3}H]Nitrendipine and [^{3}H]-PN200-110 binding. Membrane protein (50–100 μg) was incubated with [^{3}H]-(±)-nitrendipine or [^{3}H]-(+)-PN200-110 in 2 ml of 50 mM MOPS (pH 7.4) for 2–3 hr at room temperature. The binding was stopped by rapid filtration through Whatman GF/F filters. Each filter was washed five times with 5 ml of ice-cold distilled water and counted wet in 10 ml of Beckman HP/b. Competitive inhibition assays were performed with the concentrations of radioligand indicated in the figure legends, and with the concentrations of inhibitor and protein. Buffer concentrations and incubation conditions were identical to those of the direct binding assays except where indicated.

Electrophysiological experiments. Single ventricular cells were isolated from guinea pig hearts following the procedure described by Brown *et al.* (15). Whole cell voltage clamp currents were recorded using the patch clamp method of Hamill *et al.* (16). The patch pipettes had a tip resistance of 1.0 MΩ or less, and the input resistance of the cell membrane used was between 10 and 100 GΩ. The capacitive current was neutralized after forming a seal. The series resistance measured from the time constant of the capacitive transient after puncturing the membrane was 2 MΩ or less. The Ca currents were less than 2 namp and the effect of series resistance was negligible for these currents. Data were obtained from cells with Ca currents that were stable for at least 5 min to test pulses delivered at a frequency of 0.1 Hz. Cells in which Ca currents were decreasing with time or, under control conditions, in which more negative holding potentials were required to sustain the current produced by a given test potential, were discarded. Responses to drugs were measured after exposures of 5–10 min.

The experimental chamber (0.2 ml) was superfused at a rate of 2 ml/min by gravity. Ca currents were isolated by placing cells in an extracellular solution of (in mM): $CaCl_2$, 2; tetraethylammonium chloride, 135; 4-aminopyridine, 5; $MgCl_2$, 1; glucose, 10; HEPES, 10 (pH 7.4). The patch pipette contained Cs-rich internal solution of (in mM): Cs aspartate, 110; CsCl, 20; $MgCl_2$, 1; ATP, 2; EGTA, 5; HEPES, 5 (pH 7.3). These solutions provided isolation of Ca currents from other membrane currents; Na and K currents were completely eliminated. The current and voltage in response to command pulses were monitored on a storage oscilloscope. Analog data were filtered at 5 kHz, sampled at 10 kHz, and stored for subsequent analysis on a PDP 11/23 computer. The concentration-response curves were fitted to Langmuir absorption isotherms using a modified Marquardt-Levenberg nonlinear squares method (17). The inactivation-voltage curves were fit to Boltzmann distributions using the same method. All experiments were performed at room temperature (20–22°).

Results

The effects of the purified enantiomers on the binding of radiolabeled DHPs. Structures of several DHPs are shown in Fig. 1. Those enantiomers studied in this paper are (+)-PN200-110, (+)- and (−)-nimodipine, (+)- and (−)-Bay k 8644, and (+)- and (−)-202-791. The binding of ^{3}[H]-(+)-PN200-110 to guinea pig ventricular membranes is shown in Fig. 2. The apparent dissociation constant for the binding of this radioligand is 0.027 ± 0.008 nM and the binding site density (B_{max}) is 0.31 ± 0.06 pmol/mg of protein (n = 14). Values are given throughout the paper as mean ± standard deviation. Similar experiments with racemic [^{3}H]nitrendipine gave an apparent dissociation constant of 0.14 ± 0.03 nM and a B_{max} value 0.32 ± 0.06 pmol/mg of protein (n = 27). For these experiments, nonspecific labeling was defined as that not displaceable by 1 μM nitrendipine. This value of nonspecific binding was con-

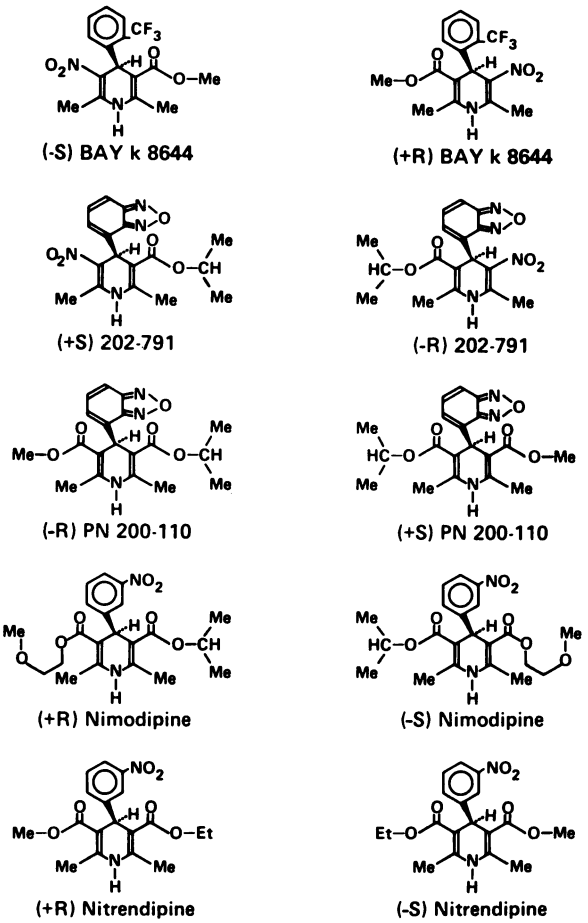

Fig. 1. Structures of the DHP enantiomers.

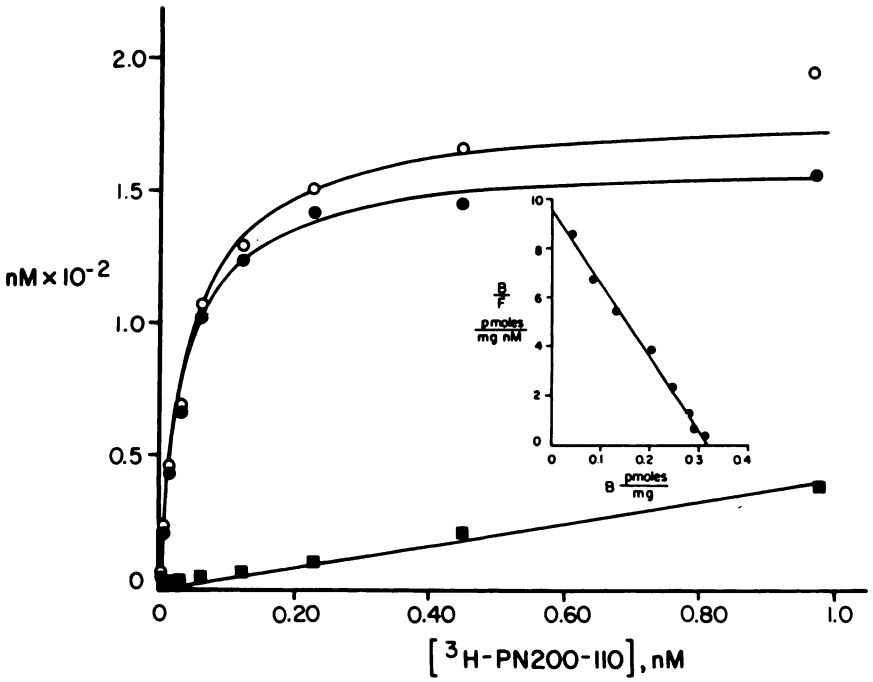

Fig. 2. [^{3}H]-(+)-PN200-110 binding to guinea pig ventricular membranes. Guinea pig ventricular membranes (90 μg of protein) were incubated in 2 ml of 50 mM MOPS (pH 7.4) with [^{3}H]-(+)-PN200-110 (eight concentrations from 0.008 nM to 0.98 nM) for 2 hr at room temperature. Bound radioactivity was separated from free by filtration on Whatman GF/F filters. For all calculations, the added concentration of [^{3}H]-(+)-PN200-110 was corrected for radioactivity bound to membranes to determine the free concentration of radioligand. ○, total pmol per mg bound; ■, amount bound in the presence of 1 μM nitrendipine; ●, specific binding. *Inset*: Scatchard analysis of specific binding.

firmed using 1 μM nimodipine, 1 μM nifedipine, and 1 μM PN200-110.

A study of the rate of binding of [^{3}H]-(±)-nitrendipine at a number of different concentrations[1] gives rise to a forward rate constant (k_1) of 6.1 × 10^8 M^{-1} min^{-1}. Measuring the rate of dissociation after the addition of 1 μM nitrendipine, we calculate an off-rate constant of 0.057 ± min^{-1} (n = 6). [^{3}H]-(+)-PN200-110 dissociates much more slowly than [^{3}H]-(±)-nitrendipine (Fig. 3B), and we calculate an off-rate constant of 0.0052 min^{-1}. Also shown in Fig. 3A is the association of [^{3}H]-PN200-110 as a function of time after the addition of membranes to a solution containing 0.2 nM [^{3}H]-(+)-PN200-110. From these data a value of 0.056 is calculated for k_{obs} at 0.2 nM [^{3}H]-PN200-110. Using the equation

$$k_{+1} = \frac{k_{obs} - k_{-1}}{[PN200\text{-}110]}$$

we calculate a forward rate constant (k_{+1}) of 2.54 × 10^8 M^{-1}. min^{-1}. The K_d, therefore, calculated from kinetic data

$$k_d = \frac{k - 1}{k_{+1}}$$

of 0.02 nM is close to the value obtained from equilibrium binding. The value of k_{-1} is not dependent on the DHP used to initiate dissociation (Table 1), indicating that there are no apparent allosteric effects on the [^{3}H]-PN200-110-binding site upon binding of either excitatory or inhibitory DHPs. It should be noted, however, that membrane vesicles are depolarized and, hence, the channel is presumably in an inactivated state. However, higher concentrations (≥10^{-5} M) of the unlabeled ligands did result in more rapid dissociation of bound [^{3}H]-(+)-PN200-110, possibly indicating nonspecific membrane perturbations at these concentrations. The difference in K_d values for [^{3}H]-(±)-nitrendipine and [^{3}H]-(+)-PN200-110 can be explained primarily by a difference in off-rates. This is in agreement with the results of Weiland and Oswald (18). We do not, however, observe the biphasic association of [^{3}H]-(±)-nitrendipine or ^{3}H-(+)-PN200-110 seen by these workers, even at higher radioligand concentrations.

In an attempt to determine the apparent K_i values for the

[1] K. L. Brush, M. Perez, M. J. Hawkes, D. R. Pratt, and S. L. Hamilton, submitted for publication.

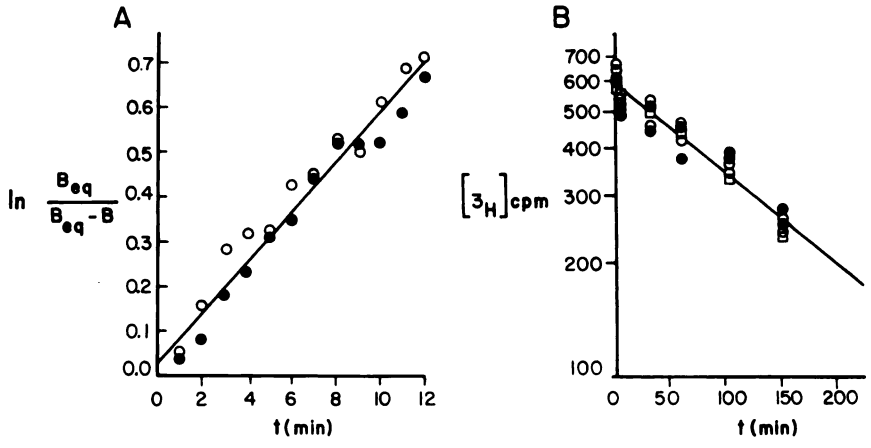

Fig. 3. Kinetics of [^{3}H]-(+)-PN200-110 binding. A. Membranes (750 μg) were added to 30 ml of 50 mM MOPS (pH 7.4) containing 0.2 nM [^{3}H]-(+)-PN200-110. Aliquots (1 ml) were filtered as a function of time after the addition of membranes. Equilibrium values were obtained after 2 hr at room temperature (26°). Nonspecific labeling was determined by identical incubations except those containing 1 μM (−)-nimodipine or 10 μM (+)-nimodipine. ○, ●, data on two different experiments. B. The rate of dissociation was calculated by filtration of 1 ml of the sample (in duplicate) as a function of time after the addition of 1 μM (−)-nimodipine (●), 10 μM (+)-nimodipine (○), or 1 μM (+)-PN200-110 (□). Nonspecific binding was followed by filtration of samples containing 1 μM cold DHP in addition to the radiolabel and was confirmed by filtration of samples at more than 10 hr after addition of excess unlabeled ligand.

TABLE 1

Dissociation rate constants for [^{3}H]-PN200-110 binding

Membranes (100 μM/ml) were incubated in 50 mM MOPS (pH 7.4) containing [^{3}H]-PN200-110 (0.2–1 nM) for 2 hr at room temperature. Dissociation was initiated by the addition of the unlabeled ligand at the concentration indicated. One-ml aliquots were filtered every 30 min for 3–10 hr.

Unlabeled ligand	k_{-1} (min)$^{-1}$	
1 μM (±)-Nimodipine	0.0040 ± 0.0014	(n = 3)
1 μM (±)-Nitrendipine	0.0052 ± 0.0016	(n = 4)
1 μM (−)-Bay k 8644	0.0052 ± 0.0008	(n = 5)
10 μM (+)-Bay k 8644	0.0053 ± 0.008	(n = 5)
1 μM (−)-Nimodipine	0.0050 ± 0.001	(n = 3)
10 μM (+)-Nimodipine	0.0056	
	0.0040	(n = 2)
1 μM (+)-PN200-110	0.0060	(n = 1)

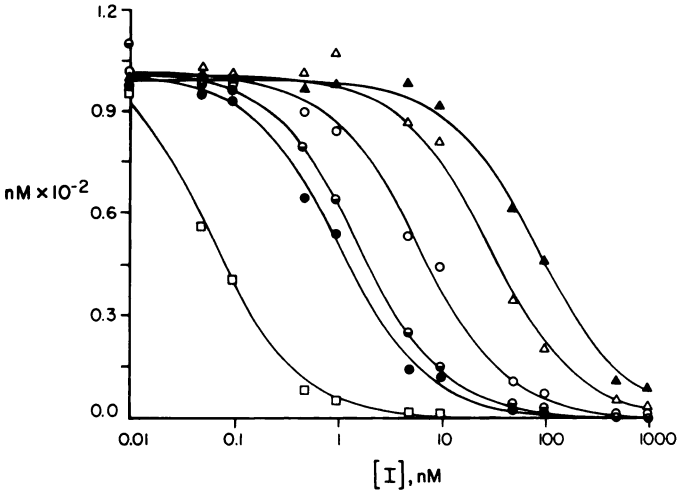

Fig. 4. Inhibition of [^{3}H]-(+)-PN200-110 binding by the DHP enantiomers. Guinea pig ventricular membranes (100 μg of protein in 2 ml of 50 mM MOPS, pH 7.4) were incubated with 0.1 nM [^{3}H]-(+)-PN200-110 in the presence of the indicated concentrations of unlabeled (+)-nimodipine (○), (−)-nimodipine (●), (±)-nimodipine (⊖) (+)-Bay k 8644 (△), (−)-Bay k 8644 (▲), or (+)-PN200-110 (□). Incubation was for 2½ hr at room temperature. Nonspecific binding was defined as that not displaceable by 1 μM nitrendipine. Filtration was through Whatman GF/F filters.

purified enantiomers of several DHPs, inhibition curves such as those shown in Fig. 4 were generated. The IC_{50} obtained from competitive displacement curves approximates the K_d of the ligand only if the concentration of labeled ligand and binding sites is small compared to the dissociation constant of the radioligand (15). This figure illustrates the inhibition of [^{3}H]-(+)-PN200-110 by the enantiomers of Bay k 8644 and nimodipine. For comparison, the inhibition by racemic nimodipine is also shown in this figure. Similar data from other experiments are summarized in Table 2. Because of the high receptor concentration (0.010–0.014 nM) used in these experiments and the high affinity of (+)-PN200-110 for this binding site, the apparent K_i values for the inhibitors were calculated from the following equation (19):

$$K_i = \frac{IC_{50}}{1 + 1/K_d(L_t + R_t - 3/2\ RL_0)}$$

In this equation K_i is the apparent dissociation constant of the competing unlabeled ligand, IC_{50} is the concentration of inhibitor which reduces the bound radioligand to one-half the amount bound in the absence of inhibitor, K_d is the apparent dissociation constant of the radioligand, L_t is the added concentration of radioligand, R_t is the total concentration of binding sites, and RL_0 is the concentration of radioligand bound in the absence of inhibitor. The K_i values calculated for (+)-Bay k 8644, (±)-Bay k 8644, (−)-Bay k 8644, (+)-nimodipine, (−)-nimodipine, and (±)-nimodipine are shown in Table 2. The effects of some of these compounds on Scatchard plots of [^{3}H]-(+)-PN200-110 binding are shown in Fig. 5. There is an apparent change in the slope of this plot with each drug but no change in the x intercept, indicating that these compounds are apparently interacting competitively with the [^{3}H]-(+)-PN200-110-binding site. Because of the problems inherent in calculating K_i values from inhibition curves (20), K_i values were also calculated from changes in the apparent K_d of [^{3}H]-(+)-PN200-110 binding determined from Scatchard plots using the following equation:

$$K_{dapp} = K_d\left(\frac{I}{K_i} + 1\right)$$

where I is the concentration of unlabeled inhibitor. When the radioligand bound was greater than 5% of the radioligand added, the concentration bound was subtracted from the added concentration to obtain the free concentration of radioligand for use in these calculations. Protein concentrations for these experiments were always less than 0.05 mg/ml. The effect of radioligand partitioning into lipids on the free concentration of radioligand is minimal at these protein concentrations. In Fig. 6 the apparent K_d of [^{3}H]-(+)-PN200-110 in the presence of (−)-Bay k 8644 is plotted as a function of the concentration of (−)-Bay k 8644. K_I values were determined from the x intercept. The K_i values calculated in this way are also summarized in Table 2. As can be seen, these values are in reasonable agreement with those obtained from inhibition curves.

DHP enantiomers with agonist effects on I_{Ca}. Both (−)-Bay k 8644 and (+)-*S*-202-791 produced large increases in I_{Ca} in guinea pig ventricular cells. The onset of action was rapid (at 10^{-6} M, the half-time was less than 30 sec) and washing the cell resulted in partial recovery. Fig. 7 shows the I_{Ca} of ventricular cells in the absence (Fig. 7Aa) and presence of (−)-Bay k 8644 at 30 nM (Fig. 7Ab), 100 nM (Fig. 7Ac), and 5 μM (Fig. 7Ad). In the presence of (−)-Bay k 8644, peak I_{Ca} increased in a concentration-dependent manner.

The concentration dependence of the effects of (−)-Bay k 8644 on I_{Ca} at 5–8 min after drug exposure is shown in Fig. 7B. The test potential was 0 or +10 mV where the I_{Ca} reaches maximum in the control conditions and holding potentials were between −80 and −40 mV. The continuous line is best fit by a one-to-one drug receptor interaction having an EC_{50} of 29 nM. As we previously reported, (+)-*S*-202-791 also produces a dose-dependent increase in I_{Ca} of ventricular cells (12). This effect is also seen in atrial cells (data not shown) and the EC_{50} is 80 nM, which is similar to the value obtained for (−)-Bay k 8644. The maximum increase in I_{Ca} by (−)-Bay k 8644 at test potential 0 or +10 mV was 3.5 ± 1-fold (n = 10) and by (+)-*S*-202-791 was 2.5 ± 1-fold (n = 8).

The magnitude of the increase in I_{Ca} by both (−)-Bay k 8644 and (+)-*S*-202-791 is larger at more negative test potentials. Fig. 8 shows the action of (−)-Bay k 8644 (100 nM) on the I-V relationship of a ventricular cell. The current records at each test potential in the absence (*open circles*) and presence (*solid circles*) is shown in Fig. 8A. The holding potential was −50 mV. In the presence of (−)-Bay k 8644, the amplitude of the peak I_{Ca} was increased at all test potentials; the drug-induced en-

TABLE 2

A comparison of the dissociation constants for binding to the inactivated state of the calcium channel with K_i values determined from radioligand binding

DHP	Radioligand	K_i[a]	K_i[b]	EC_{50} (−80 mV)	IC_{50} (−30 mV)	IC_{50} (−80 mV)	K_{av}[c]
		nM	nM				nM
A. Activators							
S-(−)-Bay k 8644	PN	4.4 ± 2.3 (n = 4)	2.3 ± 1.2 (n = 4)	29 ± 5.5 (n = 8)			
S-(+)-202-791 (Ref. 7)	N	90 ± 30 (n = 3)		80 ± 17 (n = 6)			
R/S-(±)-Bay k 8644	N	3.7 ± 1.4 (n = 10)	5.7 (n = 2)	30 ± 3 (n = 10)			
	PN	5.0 ± 2.5 (n = 8)					
B. Inhibitors							
R-(+)-Bay k 8644	PN	12.9 ± 6.8 (n = 6)	7.0 ± 7 (n = 3)		26 ± 10 (n = 5)	8000 ± 980 (n = 8)	13
R-(−)-202-791 (Ref. 7)	N	0.9 ± 0.3 (n = 3)			1.00 ± 0.95 (n = 8)	200 ± 170 (n = 8)	0.50
R-(+)-Nimodipine	PN	5.8 (n = 2)	5.33 (n = 1)		52 ± 23 (n = 5)	2000 ± 1140 (n = 6)	26
S-(−)-Nimodipine	N	0.26 ± 0.03 (n = 6)			4.0 ± 0.5 (n = 5)	450 ± 72 (n = 6)	2.0
	PN	0.17 (n = 2)	0.15 (n = 2)				
S-(+)-PN 200-110	PN	0.012 (n = 1)	0.035 (n = 1)		4.5 ± 0.7 (n = 6)	400 ± 65 (n = 6)	2.3
R/S-(±)-Nimodipine	N	0.37 ± 0.08 (n = 6)					
		0.40 (n = 2)	0.41 (n = 1)				

[a] K_i values obtained from inhibition curves, inhibition curves at the concentration of binding sites indicated were generated and curve-fitted as described in Fig. 2 using 12 concentrations of inhibitor from 10^{-11} to 10^{-6} M. Incubations were >2 hr at room temperature. The radioligand used was either [^{3}H]-(±)-nitrendipine (N) or [^{3}H]-(+)-PN200-110 (PN) at concentrations from 0.1 to 1 nM. Values are given as the mean ± standard deviation.

[b] For K_i values determined from changes in K_d apparent, K_d apparent values were as described in Figs. 3 and 4. The $-K_i$ was defined as the (x) intercept of the plot of K_{app} versus concentration of inhibitor. These plots were fitted by linear regression.

[c] K_{av} values were calculated from the IC_{50} values obtained at −30 mV and −80 mV as described in the discussion of values for the EC_{50} or IC_{50} and are given as the mean ± standard deviation.

hancement, however, was larger at negative test potentials and became smaller with increasing depolarization. The peak current amplitude was enhanced in the presence of the drug to a greater extent than the later current (see also Fig. 7). At concentrations higher than 10 nM and at holding potentials more negative than −40 mV, (−)-Bay k 8644 always caused a shift of the peak of the I-V curve to hyperpolarized potentials. However, the shift in the I-V curve was not clearly concentration dependent, and a shift of 8.0 ± 2.6 mV (n = 10) was obtained at concentrations between 10 nM and 5 μM.

Quantitatively similar results were obtained with 202-791. As in the case of (−)-Bay k 8644, (+)-*S*-202-791 always produced hyperpolarizing shifts in the I-V relationship (data not shown). The shift was 5.0 ± 1.4 mV (n = 8) at concentrations between 30 nM and 5 μM. The drug-induced enhancement of peak I_{Ca} was larger at negative test potentials, as was the case of (−)-Bay k 8644.

Previous studies suggested that the actions of Ca channel antagonists were modulated by the holding potential (3–6). To test whether this is also true for agonists, we have studied the effects of holding potential on the agonist actions over a wide range of potentials. Availability of I_{Ca} was characterized by double-pulse protocol. Fig. 9 shows a representative steady state inactivation curve for I_{Ca} in the absence (Fig. 9, *open circles*) and presence (Fig. 9, *solid circles*) of (−)-Bay k 8466. The pulse duration was 30 sec and the test potential was +10 mV. The curves were fit to the conventional inactivation-potential relation based on an assumed Boltzmann distribution (21):

$$I = I_{max}/(1 + \exp[V_m - V_h)/k])$$

where V_h is the midpotential and k is the slope of the curve. The mean control values for V_h and k were −24 ± 7 mV and 6.0 ± 1.5 mV (n = 20), respectively. In the presence of (−)-Bay k 8644, the curve shifted to negative potentials without changing the slope factor k. The increase of I_{Ca} was larger at negative potentials and became progressively smaller with increasing the holding potential. At 1 μM, the shift of V_h was 5 ± 3 mV (n = 8). The change of inactivation curve was not closely related to the drug concentrations and similar values were obtained with a concentration between 10 nM and 5 μM (−)-Bay k 8644. Qualitatively similar results were obtained with (+)-*S*-202-791, and the shift of V_h was 5 ± 4 mV (n = 5) at 1 μM.

Enantiomers of the DHPs with inhibitory effects on I_{Ca}. (+)-Bay k 8644 and (−)-*R*-202-791 had inhibitory effects on I_{Ca}. At negative holding potentials (<−50 mV) the onset of the drug action was relatively slow compared with the agonist form, and at 1 μM, the effects reached steady state 5–8 min after applying the drug. Fig. 10 illustrates the effects of (+)-Bay k 8644 on I_{Ca} at concentrations of 10 nM (Fig. 10Ab), 100 nM (Fig. 10Ac), and 1 μM (Fig. 10Ad). Fig. 10Aa represents the control in the absence of the drug.

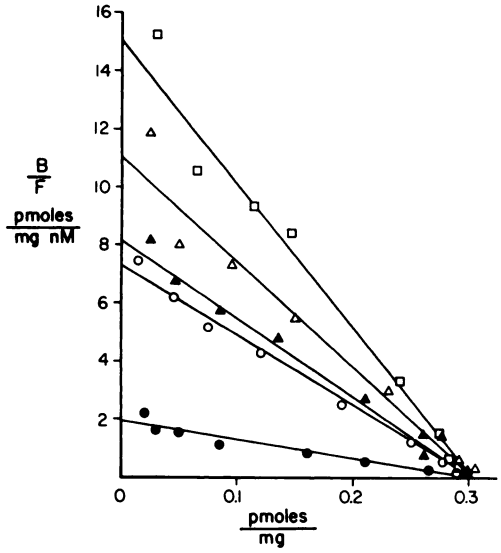

Fig. 5. Scatchard analysis of [^{3}H]-(+)-PN200-110 binding in the presence of unlabeled enantiomers. Guinea pig ventricular membranes (96 μg of protein) were incubated with [^{3}H]-(+)-PN200-110 as described in Fig. 1 in the presence of 1 nM (−)-Bay k 8644 (▲), 10 nM (+)-Bay k 8644 (△), 10 nM (+)-nimodipine (○), or 1 nM (−)-nimodipine (●). Also shown are the data obtained in the absence of inhibitor (□). Incubation was for 2.5 hr at room temperature.

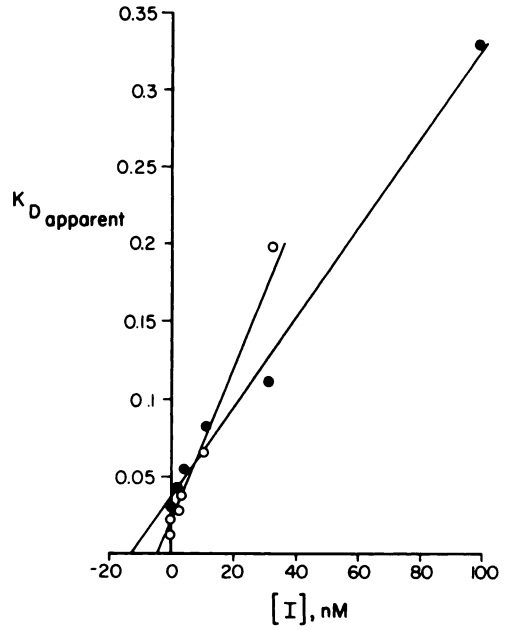

Fig. 6. Apparent K_d as a function of inhibitor concentration for the Bay k 8644 enantiomers. Guinea pig ventricular membranes (90 μg) were incubated with [^{3}H]-(+)-PN200-110 (0.03–1 nM) as in Fig. 3 in the presence of 0, 1, 3.3, 10, or 33 nM (−)-Bay k 8644 or 0, 0.1, 1, 3.3, 10, 33, or 100 nM (+)-Bay k 8644; added concentrations of [^{3}H]-(+)-PN200-110 were corrected to free by subtraction of bound concentrations. K_d apparent was calculated from a Scatchard plot of the specific binding as

$$\text{slope} = \frac{1}{-K_{app}}$$

In the presence of (+)-Bay k 8644, peak I_{Ca} was reduced in a concentration-dependent manner. The concentration dependence of the effects of (+)-Bay k 8644 on I_{Ca} for the response measured at 10–15 min after drug exposure is shown in Fig. 10B. As will be seen in Fig. 11, the (+)-Bay k 8644 block of I_{Ca} depended upon the holding potential; thus, the data were obtained at holding potentials of −30 mV (Fig. 10B, *open circles*), −50 mV (Fig. 10B, *half-open* circles), and −80 mV (Fig. 10B, *solid circles*). The test potential was 0 or +10 mV. The continuous lines were best fit by a one-to-one drug receptor interaction model with IC_{50} values of 26 nM, 730 nM, and 8 μM at −30, −50, and −80 mV, respectively. The results were qualitatively similar to those of our previous report with (−)-*R*-202-791 (12), in which the IC_{50} values at −30 and −80 mV were 1 nM and 200 nM, respectively. (+)-Bay k 8644, therefore, appears to be less potent than (−)-*R*-202-791.

Fig. 11B shows the effects of (+)-Bay k 8644, (1 μM) on the I-V relationships of the I_{Ca} of the ventricle cell. Fig. 11A shows current records at each test potential in the absence (Fig. 11A, *open circles*) and presence (Fig. 11A, *solid circles*) of the drug. The holding potential was −50 mV. (+)-Bay k 8644 decreased the amplitude of peak I_{Ca} and the effects were larger at late current. In the presence of (+)-Bay k 8644, I-V relationships of I_{Ca} remained unchanged and the current amplitude was reduced at all test potentials.

The actions of the antagonists on the Ca Channel availability were examined using the same protocol as in Fig. 9. As in the case of agonists, in the presence of (+)-Bay k 8644 or (−)-*R*-202-791, the voltage-dependent Ca channel availability curve shifted to hyperpolarized potentials. However, the shift was clearly concentration dependent. The shape of the inactivation curve was not significantly altered. The action of 1 μM (+)-Bay k 8644 on the inactivation curve is shown in Fig. 11C. In four cells the shift of V_h in the presence of (+)-Bay k 8644, at 100 nM, was 5 ± 3 mV and at 1 μM it was 10 ± 5 mV. (−)-*R*-202-791 produced a similar hyperpolarizing shift of the inactivation curve without significant change in the slope factor. However, the shift was larger than that induced by (+)-Bay k 8644, and the shift of V_h at 100 nM was 10 ± 5 mV and at 1 μM was 20 ± 3 mV.

The effects of (+)-PN200-110 and (+)- and (−)-nimodipine on I_{Ca} were also examined. These compounds blocked I_{Ca} without changing the I-V relationships; however, as with (+)-Bay k 8644 and (−)-*R*-202-791, the effects were holding potential dependent. Fig. 12A compares the current amplitudes elicited by depolarizing pulses to +10 mV from holding potentials of −80 and −30 mV, in the absence (Fig. 12Aa) and presence (Fig. 12Ab) of (+)-PN200-110 at 10 nM. At a holding potential of −80 mV, the drug showed little blocking, however at −30 mV, more than 70% of current was blocked. The concentration dependence of the drug effects was plotted in Fig. 12B using the same protocol described in Fig. 10. The IC_{50} values are 4.5 nM and 400 nM, at −30 and −80 mV, respectively. Both (−)- and (+)-nimodipine showed holding potential-dependent blocks of I_{Ca} similar to those of (+)-PN200-110. (+)-Nimodipine was, however, less potent than (−)-nimodipine. The concentration dependence of (+)- and (−)-nimodipine at holding potentials of −80 and −30 mV are shown in Fig. 12C. The IC_{50} values for (−)-nimodipine at −30 values and −80 mV were 4 nM and 450 nM, respectively. (+)-Nimodipine had IC_{50} values of 52 nM and 2 μM, at −30 mV and at −80 mV, respectively.

Discussion

The experiments carried out in this study represent an extension of our original concept (12) that the pharmacological kinetic constants should be quantitatively related to affinity

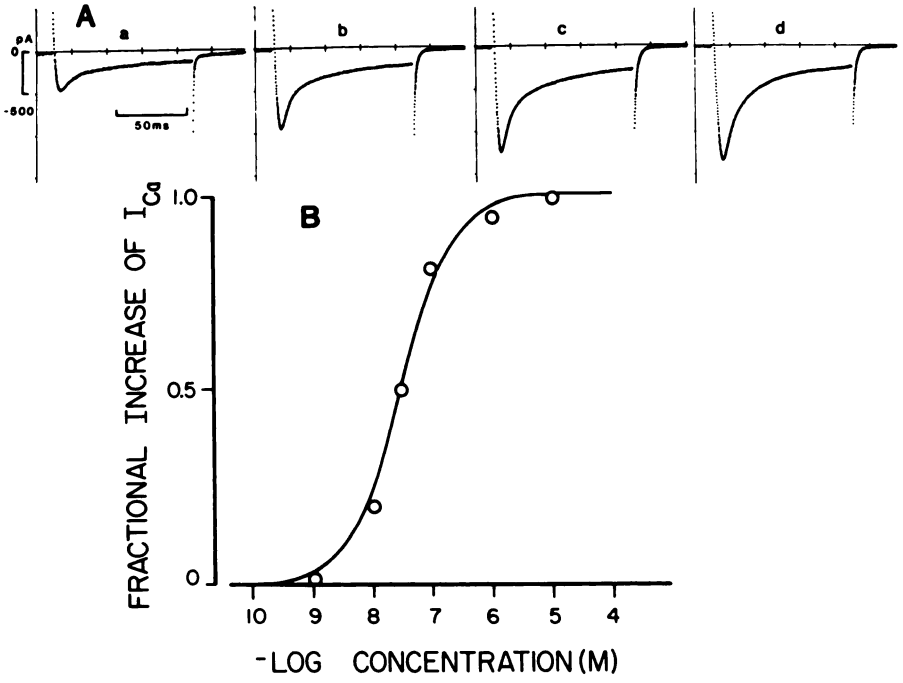

Fig. 7. Effects of (−)-Bay k 8644 on whole cell I_{Ca} of isolated guinea pig ventricle cell. A. Original current traces before (a) and after a 5-min superfusion with (−)-Bay k 9644 at 30 nM (b), 100 nM (c), and 1 μM (d). The holding potential was −50 mV and test pulses to +10 mV for 100 msec were applied at 0.1 Hz. B. Concentration-response curve for (−)-Bay k 8644. Each point represents the mean value for six to eight cells. Data were obtained at test potentials of 0 or +10 mV with holding potentials between −40 and −80 mV. Pulse rate was 0.1 Hz. ED_{50} = 29 nM.

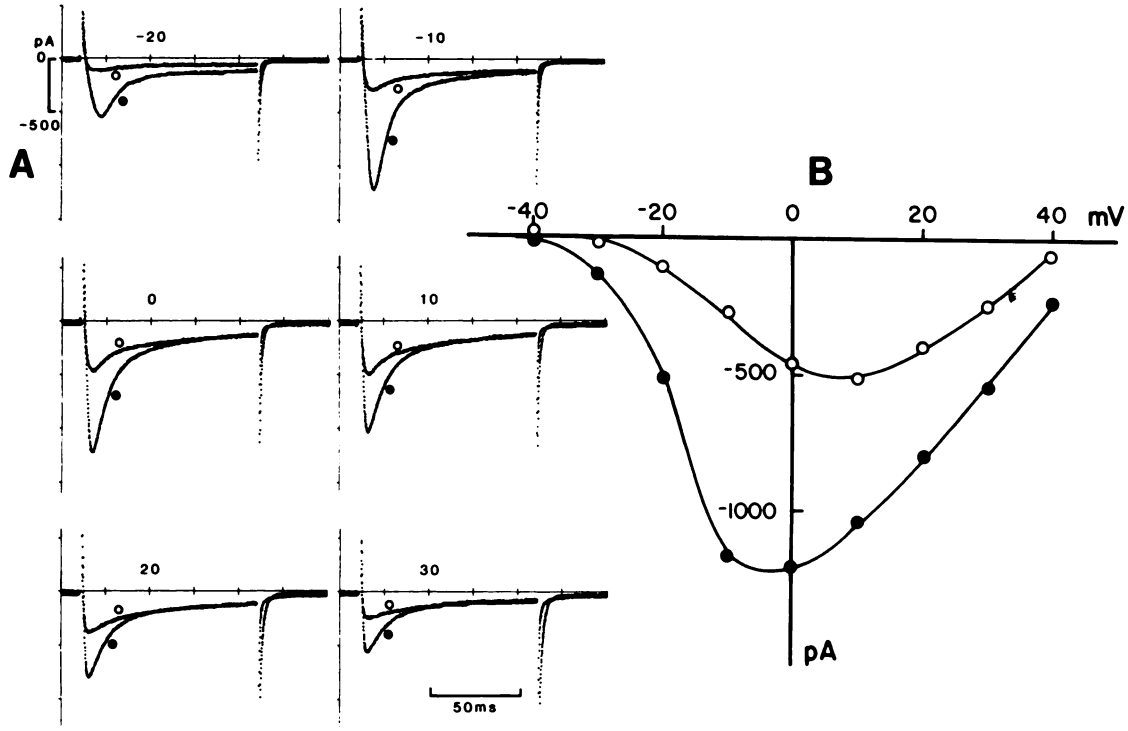

Fig. 8. Effects of (−)-Bay k 8644 (100 nM) on the I-V relationships for I_{Ca}. A shows original current traces before (○) and after (●) applying the drug at each test potential. Holding potential was −50 mV and pulse rate was 0.1 Hz. The I-V curves before (○) and after (●) (−)-Bay k 8644 were plotted in B.

constants calculated from radioligand binding data if a specific protein is believed to be a "receptor." A major problem in this area has been the 1000-fold difference between the K_d and the IC_{50} for the DHPs. The purpose of these experiments is to shed light on the reasons for the discrepancy between the concentrations of the DHPs which give rise to half-maximal electrophysiological effects and the K_d values determined from radioligand binding.

Several explanations of this peculiarity are possible. 1) Some racemic DHPs are comprised of oppositely acting enantiomers which would greatly complicate electrophysiological concentration-response data; for this reason, in this study only purified enantiomers are used. 2) The electrophysiological effects could be mediated by a low affinity site or some other receptor different from the high affinity site which has been characterized by radioligand binding techniques. 3) The affinity of the DHPs for the binding site is dependent upon the state (conformation) of the receptor which is in itself dependent on mem-

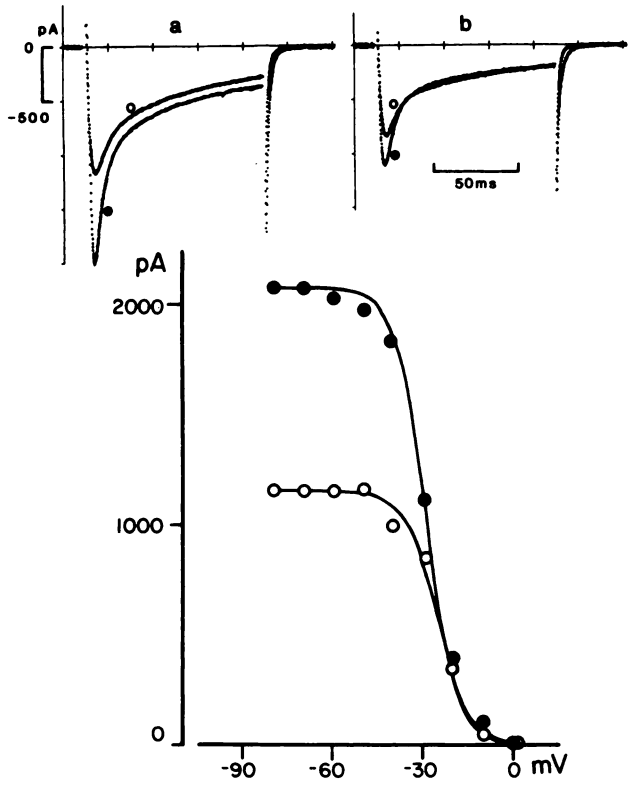

Fig. 9. Effects of (−)-Bay k 8644 (100 nM) on voltage dependence of I_{Ca} availability. Prepulses of 30 sec in duration were followed by a 100-msec test pulse to +10 mV from a holding potential of −50 mV. *Solid lines* were drawn according to $I = I_{max}/(1 + \exp[(V_m - V_h/k)])$, where V_h is the midpotential of the curve and k is the slope of the curve. For the control (○), V_h = −25.6 mV and k = 5.3 mV; for (−)-Bay k 8644 (●), V_h = −29 mV and k = 5.0 mV. The *top* (a, b) shows superimposed current traces during the test pulse at +10 mV before (○) and after (●) the drug. a, prepulses to −50 mV; b, prepulses to −30 mV.

brane potential; therefore, major differences would be found between membrane fragments which are depolarized and intact cells which are polarized. 4) Each DHP may have unique effects on calcium channel gating. 5) Some combination of all or part of these effects contributes to the differences between radioligand binding and electrophysiological response. These and other complications have been discussed and reviewed by Schwartz and his colleagues (2, 10).

Low affinity binding sites for the DHPs have been reported by several laboratories (7–10, 22–28).[2] Although some of these sites are unrelated to calcium channels (27–30), there is some evidence for a functional low affinity DHP-binding site on calcium channels. At negative holding potentials the concentration-response curve for both (±)-nitrendipine and (±)-Bay k 8644 is best fit by a Langmuir adsorption isotherm model which is the sum of two independent one-to-one drug receptor sites (6). From single channel analysis the primary effect of high concentrations (1 μM or greater) of Bay k 8644 is an increase in the average open times of the calcium channels (31, 32). In contrast, lower concentrations increased calcium currents primarily by increasing the probability of opening (15).

Generally not considered in the models to explain the differences between DHP binding and concentration-response curves is the observation that some DHPs activate rather than inhibit voltage-dependent calcium channels. As shown in this paper and from other reports (5, 6, 7, 8, 11, 12, 13, 29, 33–35), two such compounds are *S*-(−)-BAY k 8644 and *S*-(+)-202-791. The EC_{50} for *S*-(+)-202-791 is reasonably close to the K_d value obtained from radioligand binding (12); for *S*-(−)-Bay k 8644, however, these values differ by an order of magnitude. Part of this discrepancy could be due to a small amount of contamination with the (+)-enantiomer, which is inhibitory. *S*-(+)-202-791 is less potent at activating calcium current than is *S*-(−)-Bay k 8644. *S*-(+)-202-791 and *S*-(−)-Bay k 8644 are probably both partial agonists, as suggested in several communications (5–10). The agonist efficacy of these compounds

[2] K. L. Brush, M. Perez, M. J. Hawkes, D. R. Pratt, and S. L. Hamilton, submitted for publication.

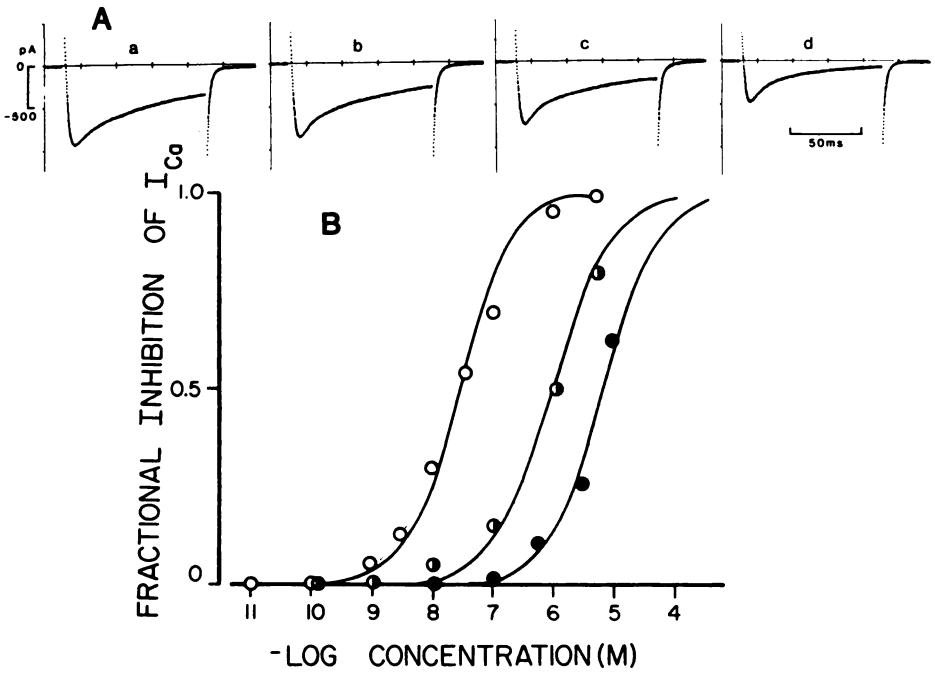

Fig. 10. Effects of (+)-Bay k 8644 on I_{Ca}. A. Original current traces before (a) and after a 5-min superfusion with (+)-Bay k 8644 at 10 nM (b), 100 nM (c), and 1 μM (d). The holding potential was −50 mV and test pulses to 0 mV for 100 msec were applied at 0.1 Hz. B. Concentration-response curves for (+)-Bay k 8644 at holding potentials of either −80 or −90 mV (●), −50 mV (◒), and −30 mV (○). Each point represents the mean value for five to eight cells. The test potentials are 0 or +10 mV. IC_{50} values are 26 nM, 730 nM, and 8 μM, at −30, −50, and −80 or −90 mV, respectively. At negative potentials complete block was not obtained.

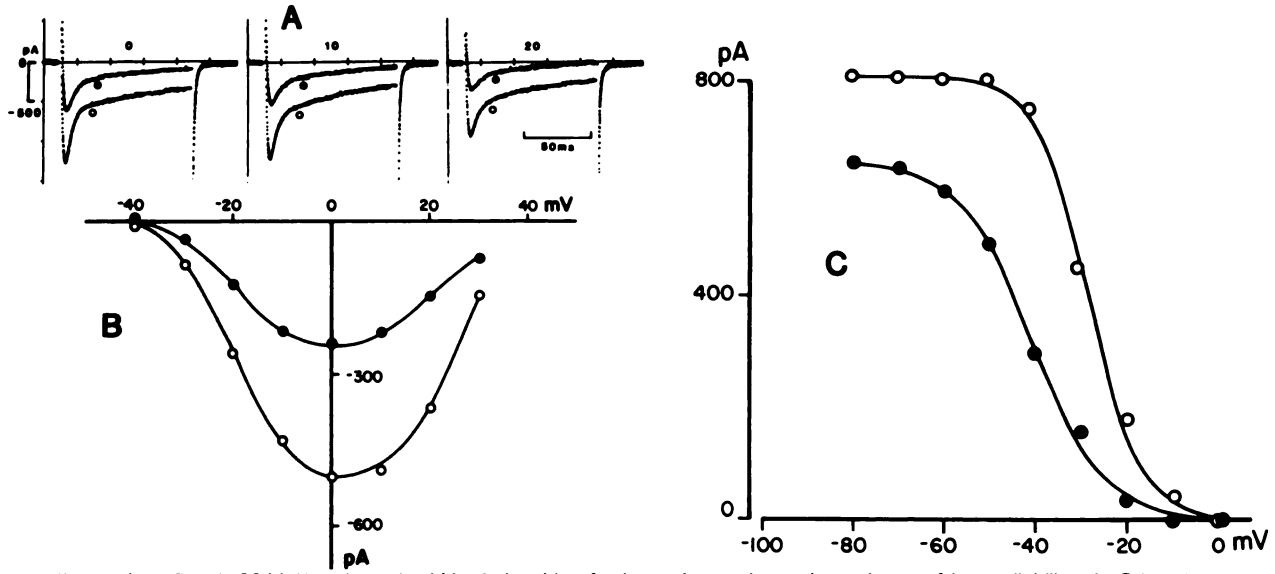

Fig. 11. Effects of (+)-Bay k 8644 (1 μM) on the I-V relationships for I_{Ca} and on voltage dependence of I_{Ca} availability. A. Original current traces before (O) and after (●) the drug at each test potential. B. Plot of the I-V curves before (O) and after (●) (+)-Bay k 8644. C. Ca channel availability curves in the absence (O) and presence (●) of the drug. Prepulse duration was 30 sec and test potential was +10 mV. *Solid lines* were drawn as in Fig. 9. For control, $V_h = -28$ mV, $k = 6.0$ mV. With (+)-Bay k 8644, $V_h = -40$ mV, k = 7.7 mV.

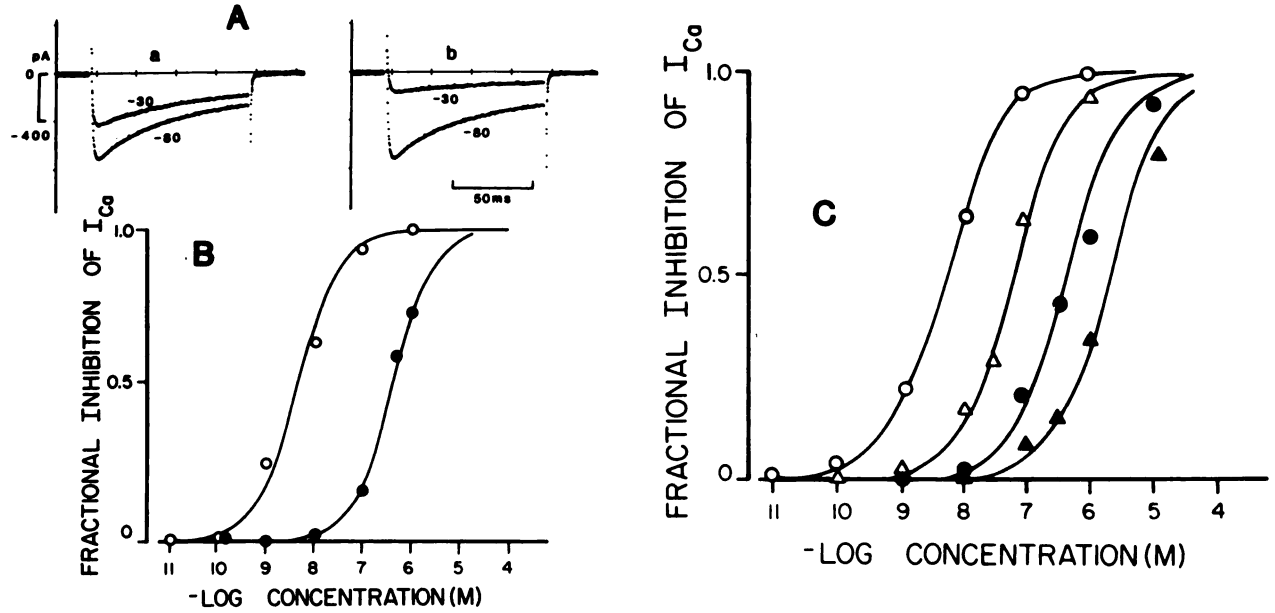

Fig. 12. Effects of (+)-PN200-110 and (−), (+)-nimodipine on I_{Ca}. The *inset* in A shows current traces before (a) and after (b) (+)-PN200-110, 10 nM. Currents elicited by depolarizing pulses to + 10 mV from holding potentials of −80 and −30 mV were superimposed. The concentration-response curves for (+)-PN200-110 at holding potentials of −80 mV (●) and −30 mV (O) are shown in B. Each point represents the mean value for four to six cells. Test potentials were 0 or +10 mV. IC_{50} values are 4.5 nM and 400 nM, at −30 and −80 mV, respectively. For (−)-nimodipine, IC_{50} values are 450 nM and 4 nM, at −80 and −30 mV, respectively. C shows concentration-response curves for (−)-nimodipine (O, ●) and (+)-nimodipine (Δ, ▲) at −30 mV (O, Δ) and −80 mV (●, ▲). For (+)-nimodipine, IC_{50} values are 52 nM and 2 μM, at −30 and −80 mV, respectively. For (−)-nimodipine, the IC_{50} values are 4 nM and 450 nM, respectively. At negative potential, complete block was not obtained.

is greater at more negative test potentials and at more negative holding potentials; the potency, however, does not appear to be affected by the holding potentials between −40 and −80 mV (Fig. 9). The lack of voltage dependence of the potency of their effects suggests that the affinity of these compounds for the rested state versus the states assumed by the channel upon depolarization is approximately equal over these potentials. We cannot, however, evaluate potency at more depolarized holding potentials (Fig. 9). Since the efficacy of the agonists becomes much less as the membranes become more depolarized, small changes in the potency become more difficult to measure. The binding studies suggest that, at least for *S*-(−)-Bay k 8644, the drug may have a higher affinity for the inactivated state. Although our original study (12) showed no voltage dependence (Fig. 2 of Ref. 12), the data in Fig. 9 suggest that both (+)-202-791 and (−)-Bay k 8644 may have some voltage dependence. The structural features of the DHPs which give rise to inhibitory versus excitatory activity remain to be elucidated. Most binding and pharmacological data indicate that the two types of dihydropyridines share a common binding site (7–10, 12, 35–

39). In agreement with this, in the current paper we show that unlabeled agonist and antagonist alter the apparent K_d but not the Bmax for [^{3}H]-PN200-110 binding and that there is no effect of the different DHPs on the rate of [^{3}H]PN200-110 dissociation.

At least part of the discrepancy for binding versus electrophysiological data for the inhibitory DHPs can be explained by the voltage dependence of the block by these DHPs (3–5) which is interpreted to indicate that the DHPs bind with higher affinity to inactivated calcium channels than to rested channels.

A number of laboratories have investigated the voltage dependence of radiolabeled DHP binding (40–43). In skeletal muscle the effect of depolarization on DHP binding appears to be a change in the B_{max} for nitrendipine binding with no change in affinity (41). This also appears to be true in isolated myocytes (40). The kinetic results of Schilling and Drew (42), however, are more consistent with a change in K_d but do not exclude a change in B_{max}. An interconversion between high and low affinity binding sites could possibly explain the differences in these observations. In this regard, Weiland and Oswald (18) have presented kinetic evidence for two interconvertible binding states for DHPs in rat brain membranes, one of high affinity and a second with very low affinity for these ligands. How these sites in isolated membranes may be affected by membrane potentials is, however, not know.

The voltage dependence of the effects of inhibitory DHPs is confirmed in this paper using (+)-nimodipine, (−)-nimodipine, (+)-Bay k 8644, (+)-PN200-110, and (−)-*R*-202-791. All of these DHPs block calcium currents at all test potentials, and the potency of block is dependent upon the holding potential. The IC_{50} values for each of these drugs at holding potentials of −80 mV (where essentially all of the channels are in the rested state) and −30 mV (where ≈ 50% are inactivated) are summarized in Table 2. To calculate the affinity of the ligands for the inactivated state we used the following equation:

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

as described by Bean (3), where h is the fraction of channels in the resting state, K_R is the dissociation constant for ligand binding to the rested state which is estimated by K_{app} at −80 mV, K_{IN} is dissociation constant for binding to the inactivated state, and K_{app} is the concentration of drug giving rise to half-maximal inhibition at −30 mV ($h \approx 0.5$). In this calculation, binding to open channels is omitted. It is probably less than that for stimulating DHPs since the time course of inactivation is unchanged even at concentrations of 1 μM (Fig. 10). The K_{IN} values calculated in this way and the average K_I values from binding experiments are shown in Table 2.

As can be seen in Table 2, preferential binding of certain inhibitory DHPs to the inactivated state of the calcium channel can explain the differences between the IC_{50} and the K_d values determined by radioligand binding. This state dependence of binding can be used to calculate a dissociation constant for binding to inactivated channels which agrees with the K_d determined from radioligand binding. This suggests that the binding studies are relevant to the physiological state. It must be noted, however, that K_d values are equilibrium values whereas the physiological results are steady or almost steady state values. Hence a comparison of kinetics might be more valid, but this has not yet been done. In the case of racemic nitrendipine a reasonable correlation of kinetics was found (3). The discrepancies between the IC_{50} and the K_d values calculated from binding for certain DHPs cannot be completely reconciled by differences in membrane potential. The major discrepancy appears to occur with (+)-PN200-110. Cognard *et al.* (44), however, in skeletal muscle found K_d values which are very close to the $K_{0.5}$ for this ligand interacting with the inactivated state. This may reflect a difference between cardiac and skeletal muscle or, if the block by PN200-110 develops slowly, our calculated value of K_{IN} may be overestimated. In this regard it is of interest that these discrepancies are not present for diltiazem and verapamil derivatives which are sterically linked to the DHP receptor.[3]

As noted, the model of high affinity binding to the inactivated state which we have used cannot explain the agonist effect of DHPs nor the fact that blockers prolong channel open time (31, 32). An alternative is that each DHP also has unique effects on the gating of calcium channels. Three other models of this sort have been proposed. One which we call the mode model (32) attributes the mixed effects to low and high affinity binding sites that stabilize the channel in one of several modes. A second, simpler model (45) proposes that DHPs bind preferentially to the open state and slow only the transition rate from the open to the closed state by an order of magnitude. A model similar to this one (46) does not limit the DHP effects to a single transition. We are presently evaluating these models and their relation to our binding and electrophysiological data.

Acknowledgments

We should like to sincerely thank Drs. William Schilling, Diana L. Kunze, Carl J. Johnson, and Pal Vaghy for their critical examination of the manuscript and their helpful advice.

References

1. Tsien, R. W. Calcium channels in excitable cell membranes. *Annu. Rev. Physiol.* **45**:341–358 (1983).
2. Schwartz, A., and D. Triggle. Cellular action of calcium channel blocking drugs. *Annu. Rev. Med.* **35**:325–329 (1984).
3. Bean, B. P. Nitrendipine block of cardiac calcium channels: high affinity binding to the inactivated state. *Proc. Natl. Acad. Sci. USA* **81**:6388–6392 (1984).
4. Sanguinetti, M. C., and R. S. Kass. Voltage-dependent block of calcium channel current in the calf cardiac Purkinje fiber by dihydropyridine calcium channel antagonists. *Circ. Res.* **55**:336–348 (1984).
5. Sanguinetti, M. C., and R. S. Kass. Regulation of cardiac calcium channel current and contractile activity by the dihydropyridine Bay k 8644 is voltage-dependent. *J. Mol. Cell. Cardiol.* **16**:667–670 (1984).
6. Brown, A. M., D. L. Kunze, and A. Yatani. Dual effects of dihydropyridines on whole cell and unitary calcium currents in single ventricular cells of guinea pig. *J. Physiol. (Lond.)*, in press, (1986).
7. Schwartz, A., I. L. Grupp, J. S. Williams, and P. L. Vaghy. Effects of dihydropyridine calcium channel modulators in the heart: pharmacological and radioligand binding correlations. *Biochem. Biophys. Res. Commun.* **125**:387–394 (1984).
8. Vaghy, P. L., I. L. Grupp, G. Grupp, and A. Schwartz. Effects of Bay k 8644, a dihydropyridine analog, on [^{3}H]nitrendipine binding to canine cardiac sarcolemma and the relationship to a positive inotropic effect. *Circ. Res.* **55**:549–553 (1984).
9. Vaghy, P. L., I. L. Grupp, G. Grupp, J. L. Blwierczak, J. S. Williams, and A. Schwartz. Correlation of nitrendipine and Bay k 8644 binding to isolated canine heart sarcolemma with their pharmacological effects on the canine heart. *Eur. J. Pharmacol.* **102**:373–374 (1984).
10. Vaghy, P. L., Galube, I. Grupp, G. Grupp, J. Williams, J. Baik, and A. Schwartz. A proposed pharmacological role for dihydropyridine binding sites in heart and coronary smooth muscle, in *Bayer-Symposium IX. Cardiovascular Effects of Dihydropyridine-Type Calcium—Antagonists and Agonists* (A. Fleckenstein, C. Van Breeran, R. Grob, and F. Hoffmeister, eds.). Springer-Verlag, Berlin, 156–184 (1985).
11. Hof, P. R., U. T. Ruegg, A. Hof, and A. Vogel. Stereoselectivity at the calcium

[3] Unpublished observations.

channel: opposite action of enantiomers of a 1,4-dihydropyridine. *J. Cardiovasc. Pharmacol.* **7**:689–693 (1985).
12. Williams, J. S., I. L. Grupp, G. Grupp, P. L. Vaghy, L. Dumont, A. Schwartz, A. Yatani, S. L. Hamilton, and A. M. Brown. Profile of the oppositely acting enantiomers of the dihydropyridine 202-791 in cardiac preparations: receptor binding, electrophysiological, and pharmacological studies. *Biochem. Biophys. Res. Commun.* **131**:13–21 (1985).
13. Franckowiak, G., M. Becham, M. Schramm, and G. Thomas. The optical isomers of the 1,4 dihydropyridine BAY K 8644 show opposite effects on Ca channels. *Eur. J. Pharmacol.* **114**:223–226 (1985).
14. Hof, R. P., A. Hof, U. T. Ruegg, N. S. Cook, and A. Vogel. Stereoselectivity at the calcium channel: different profiles of hemodynamic activity of the enantiomers of the dihydropyridine derivative PN200-110. *J. Cardiovasc. Pharmacol.* **8**:221–226 (1986).
15. Brown, A. M., D. L. Kunze, and A. Yatani. The agonist effect of dihydropyridines on Ca channels. *Nature* (*Lond.*) **311**:570–572 (1984).
16. Hamill, O. P., A. Marty, E. Neher, B. Sakmann, and F. J. Sigworth. Improved patch clamp techniques for high resolution current recordings from cells and cell-free membrane patches. *Pfluegers Arch.* **391**:85–100 (1981).
17. Bevington, P. R. *Data Reduction and Error Analysis for the Physical Sciences.* McGraw-Hill, New York. 336 (1969).
18. Weiland, G. A., and R. E. Oswald. The mechanism of binding of dihydropyridine calcium channel blockers to rat brain membranes. *J. Biol. Chem.* **260**:8456–8464 (1985).
19. Chang, K. J., S. Jacobs, and P. Cuatrecasas. Quantitative aspects of hormone-receptor interactions of high affinity. Effect of receptor concentration and measurement of dissociation constants of labeled and unlabeled hormones. *Biochim. Biophys. Acta* **406**:294–303 (1975).
20. Jacobs S., K. J. Chang, and P. Cuatrecasas. Estimation of hormone receptor affinity by competitive displacement of labeled ligand: effect of concentration of receptor and of labeled ligand. *Biochem. Biophys. Res. Commun.* **66**:687–692 (1975).
21. Hodgkin, A. L., and A. F. Huxley. A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol.* (*Lond.*) **117**:500–544 (1952).
22. Bellemann, P., D. Ferry, F. Lübbecke, and H. Glossman. [^{3}H]-Nitrendipine, a potent calcium antagonist, binds with high affinity to cardiac membranes. *Arzneim. Forsch.* **31**:2064–2067 (1981).
23. Marsh, J. D., E. Loh, D. Lachance, W. H. Barry, and T. W. Smith. Relationship of binding of a calcium channel blocker to inhibition of contraction in intact cultured embryonic chick ventricular cells. *Circ. Res.* **53**:539–543 (1983).
24. Schilling, W. P., S. L. Hamilton, A. Yatani, K. Brush, and A. M. Brown. High and low affinity nitrendipine binding in membrane preparations from guinea pig ventricle: correlation with inhibition of calcium current. *Biophys. J.* **47**:264a (1985).
25. Triggle, C. R., P. K. Agrawal, G. T. Bolger, E. E. Daniel, C. Y. Kwan, E. M. Luchowski, and D. J. Triggle. Calcium-channel antagonist binding to isolated vascular smooth muscle membranes. *Can. J. Physiol. Pharmacol.* **60**:1738–1741 (1982).
26. Litzinger, M. J., and D. E. Brennaman. [^{3}H]-Nitrendipine binding in developing dissociated fetal mouse spinal cord neurons. *Biochem. Biophys. Res. Commun.* **127**:112–119 (1985).
27. Striessnig, J., G. Zernig, and H. Glossmon. Ca^{+2} antagonist receptor sites on human red blood cell membranes. *Eur. J. Pharmacol.* **108**:329–330 (1985).
28. Kunze, D. L., M. J. Hawkes, S. L. Hamilton, and A. M. Brown. Binding and pharmacological studies of nitrendipine on PC12 cells. *Biophys. J.* **47**:264a (1985).
29. Yatani, A., and A. M. Brown. The calcium channel blocker nitrendipine blocks sodium channels in neonatal rat cardiac myocytes. *Circ. Res.* **57**:868–875 (1985).
30. Hume, J. R. Comparative interactions of organic Ca^{++} channel antagonists with myocardial Ca^{++} and K^+ channels. *J. Pharmacol. Exp. Ther.* **234**:134–140 (1985).
31. Kokobun, S., and H. Reuter. Dihydropyridine derivatives prolong the open state of Ca channels in cultured cardiac cells. *Proc. Natl. Acad. Sci. USA* **81**:4824–4827 (1984).
32. Hess, P., J. B. Lansman, and R. W. Tsien. Different modes of Ca channel gating behaviour favoured by dihydropyridine Ca agonists and antagonists. *Nature* (*Lond.*) **311**:538–544 (1984).
33. Schramm, M., G. Thomas, R. Towart, and G. Franckowiak. Novel dihydropyridines with positive inotropic action through activation of Ca^{2+} channels. *Nature* (*Lond.*) **303**:535–537 (1983).
34. Schramm, M., G. Thomas, R. Towart, and G. Franckowiak. Activation of calcium channels by novel 1,4-dihydropyridines. A new mechanism for positive inotropics or smooth muscle stimulants. *Arzneim. Forsch.* **33**:1268–1272 (1983).
35. Janis, R. A., J. G. Sarmiento, S. C. Maurer, G. T. Bolger, and D. J. Triggle. Characteristics of the binding of [^{3}H]-nitrendipine to rabbit ventricular membranes: modification by other Ca^{2+} channel antagonists and by Ca^{2+} agonist BAY K 8644. *J. Pharmacol. Exp. Ther.* **231**:8–15 (1984).
36. Janis, R. A., D. Rampe, J. G. Sarmiento, and D. J. Triggle. Specific binding of a calcium channel activator, [^{3}H] Bay k 8644, to membranes from cardiac muscle and brain. *Biochem. Biophys. Res. Commun.* **121**:317–323 (1984).
37. Rampe, D., R. A. James, and D. J. Triggle. BAY K 8644, a 1,4 dihydropyridine Ca^{2+} channel activator: dissociation of binding and functional effects in brain synaptosomes. *J. Neurochem.* **43**:1688–1692 (1985).
38. Su, C. M., V. C. Swamy, and D. J. Triggle. Calcium channel activation in vascular smooth muscle by BAY K 8644. *Can. J. Physiol. Pharmacol.* **62**:1401–1410 (1984).
39. Triggle, D. J., and R. A. Janis. The 1,4 dihydropyridine receptor: a regulatory component of the Ca^{2+} channel. *J. Cardiovasc. Pharmacol.* **7**:5949–5955 (1984).
40. Green, F. J., B. B. Farmer, G. L. Wiseman, M. J. Jose, and A. M. Watanabe. Effect of membrane depolarization on binding of [^{3}H]-nitrendipine to rat cardiac myocytes. *Circ. Res.* **56**:576–585 (1985).
41. Schwartz, L. M., E. W. McClesky, and W. Almers. Dihydropyridine receptors in muscle are voltage-dependent but most are not functional calcium channels. *Nature* (*Lond.*) **314**:747–751 (1985).
42. Schilling, W., and J. Drew. Voltage-sensitive nitrendipine binding in an isolated cardiac sarcolemma preparation. *J. Biol. Chem.* **261**:2750–2758 (1986).
43. Reuter, H., H. Porzig, S. Kokubun, and B. Prod'hom. 1,4-Dihydropyridines as tools in the study of Ca^{2+} channels. *Trends Neurosci.* **8**:396–400 (1985).
44. Cognard, C., G. Romey, J.-P. Galizzi, M. Fosset, and M. Lazdunski. Dihydropyridine-sensitive Ca^{2+} channels in mammalian skeletal muscle cells in culture: electrophysiological properties and interactions with Ca^{2+} channel activator (Bay k 8644) and inhibitor (PN200-110) *Proc. Natl. Acad. Sci. USA* **83**:1518–1522 (1986).
45. Sanguinetti, M. C., and R. S. Kass. Voltage selects activity of the Ca channel modulator Bay K 8644. *Biophys. J.* **47**:513a (1985).
46. Bechem, M., and M. Schwamm. Calcium agonists. *J. Mol. Cell. Cardiol.*, in press.

Send reprint requests to: Dr. Susan L. Hamilton, Department of Physiology and Molecular Biophysics, Baylor College of Medicine, Houston, TX 77030.