Internal and External Effects of Dihydropyridines in the Calcium Channel of Skeletal Muscle

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ABSTRACT The agonist effect of the dihydropyridine (DHP) (−)Bay K 8644 and the inhibitory effects of nine antagonist DHPs were studied at a constant membrane potential of 0 mV in Ca channels of skeletal muscle transverse tubules incorporated into planar lipid bilayers. Four phenylalkylamines (verapamil, D600, D575, and D890) and *d-cis*-diltiazem were also tested. In Ca channels activated by 1 μM Bay K 8644, the antagonists nifedipine, nitrendipine, PN200–110, nimodipine, and pure enantiomer antagonists (+)nimodipine, (−)nimodipine, (+)Bay K 8644, inhibited activity in the concentration range of 10 nM to 10 μM. Effective doses (ED_{50}) were 2 to 10 times higher when DHPs were added to the internal side than when added to the external side. This sidedness arises from different structure-activity relationships for DHPs on both sides of the Ca channel since the ranking potency of DHPs is PN200–110 > (−)nimodipine > nifedipine ~S207–180 on the external side while PN200–110 > S207–180 > nifedipine ~(−)nimodipine on the internal side. A comparison of ED_{50}'s for inhibition of single channels by DHPs added to the external side and ED_{50}'s for displacement of [^{3}H]PN200–110 bound to the DHP receptor, revealed a good quantitative agreement. However, internal ED_{50}'s of channels were consistently higher than radioligand binding affinities by up to two orders of magnitude. Evidently, Ca channels of skeletal muscle are functionally coupled to two DHP receptor sites on opposite sides of the membrane.

INTRODUCTION

Dihydropyridines (DHPs) are high affinity ligands of the Ca channel that specifically bind to a single polypeptide with a molecular weight of 175,000, i.e., the α_1 subunit of the DHP receptor (Galizzi et al., 1986; Striessnig et al., 1986; Sieber et al., 1987; Sharp et al., 1987). Two other groups of Ca antagonists, phenylalkylamines (verapamil derivatives) and benzothiazepines (diltiazem) are also bound specifically to the α_1 subunit (Galizzi et al., 1986; Sieber et al., 1987). The primary sequence of the α_1 subunit of the DHP receptor revealed a significant homology to that of the voltage-gated Na channel, which led to the suggestion that the DHP receptor and the Ca channel are the same or closely associated proteins (Tanabe et al., 1987; Ellis et al.,

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/90/01/0001/27 $2.00
Volume 95 January 1990 1–27

1988). Three additional polypeptides that copurified with α_1 as part of the DHP receptor have approximate molecular weights α_2 (170,000), β (52,000), and γ (31,000) (Takahashi et al., 1987; Catterall et al., 1988; Campbell et al., 1988; Hosey and Lazdunski, 1988; Leung et al., 1988). Evidence that DHP receptors represent functional Ca channels has been provided by single-channel recordings of purified DHP receptor preparations in planar bilayers (Flockerzi et al., 1986; Smith et al., 1987) and ^{45}Ca fluxes in liposomes (Curtis and Catterall, 1986; Horne et al., 1988). However, the minimal polypeptide composition necessary to reconstitute functional Ca channels varies widely among reports, thus, whether α_1 is the only structural component of the DHP-sensitive Ca channel is not known.

The electrophysiological approach towards establishing the relation between Ca channels and DHP receptors has been to analyze quantitatively the pharmacological effects of DHPs. In intestinal smooth muscle, there is essentially a 1:1 correlation between the competitive inhibition of [^{3}H]nitrendipine binding and the inhibition of the contractile response produced by a large number of structurally different DHPs (Mannhold et al., 1982; Bolger et al., 1983; Sarmiento et al., 1984; Su et al., 1985). This suggested a tight structural coupling between Ca channels and drug receptors in this tissue. Cardiac and skeletal muscle Ca channels are less sensitive to DHPs than expected from radioligand binding experiments (Lee and Tsien, 1983; Janis et al., 1984*b*, *c*, 1985; Palade and Almers, 1985; Schwartz et al., 1985; Williams et al., 1985; Kokubun et al., 1987; Lacerda and Brown, 1988). Recently, Hamilton et al., (1987) showed in cardiac Ca channels that numerical discrepancies between electrophysiological and radioligand binding affinities are larger at rest than at depolarized potentials. This has been interpreted to indicate that DHPs bind with higher affinities to Ca channels inactivated as a consequence of depolarization than to channels in their rest state. Similar conclusions were put forward earlier by others (Bean, 1984; Sanguinetti and Kass, 1984).

In skeletal muscle, a correlation between occupancy of the DHP receptor and sensitivity of the Ca channel to the same DHPs has not been described and we intend to do so in the present report. Recordings were made in purified rabbit transverse tubules incorporated into planar lipid bilayers to take advantage of the fact that in this system the agonist DHP Bay K 8644 promotes a steady-state activity of Ca channels at 0 mV (Affolter and Coronado, 1985, 1986; Coronado and Affolter, 1986*a*, *b*; Ehrlich et al., 1986; Rosenberg et al., 1986; Coronado and Smith, 1987; Ma and Coronado, 1988*a*, *b*; Vilven et al., 1988; Vilven and Coronado, 1988; Valdivia and Coronado, 1988, 1989; Yatani et al., 1988). This permitted the construction of dose-response curves for antagonist DHPs (in the presence of Bay K 8644) and a comparison with radioligand binding affinities of DHPs. The former were measured from single-channel records and the latter from specific displacement of [^{3}H]PN200–110 bound to the DHP receptor. Both sets of data were obtained under strictly identical conditions in the same preparation of purified rabbit transverse tubules. To our knowledge, this type of comparison has not been possible in the past. Even though binding experiments in cells can be done under stationary conditions with equilibration of the free and ligand-bound receptor (Green et al., 1985; Schwartz et al., 1985; Kokubun et al., 1987), Ca channel measurements cannot. Ca currents of cells are measured using pulse protocols during which chan-

nels open transiently and inactivate on a time scale of milliseconds even in the presence of agonist DHPs (Rosenberg et al., 1986; Lacerda and Brown, 1988; Tsien et al., 1988). Our results indicate a strong quantitative correlation between the pharmacology of single Ca channels and that of DHP receptors for a set of ten DHPs, racemic and pure enantiomers. Furthermore, internal and external effects of DHPs studied separately suggested the presence of two drug receptor sites, a low affinity site on the inside and a high affinity site on the outside. Single-channel affinities and binding affinities are essentially the same in the high affinity site. This strongly suggests that the DHP-sensitive Ca channel and DHP receptor may share common components or that they may represent different domains of the same structure.

MATERIALS AND METHODS

Preparation of Skeletal Muscle Transverse Tubules

Transverse tubule vesicles were prepared from rabbit back and leg white muscle by a modification of the microsome fractionation procedure of Meissner (1984). Light muscle microsomes sedimenting at 10%/20% sucrose interface were used in all experiments. Portions of back and leg muscle are partially homogenized in buffer A (0.3 M sucrose, 20 mM HEPES-Tris, pH 7.2) with four 15-s pulses in a food processor. Tissue is completely homogenized in 3 vol of buffer A at high speed (2 × 30 s) in a Waring blender (Waring Products Div., New Hartford, CT). The total homogenate is centrifuged for 30 min at 2,600 *g* (4,000 rpm) in a GSA-Sorvall rotor (Sorvall Instruments Div., Newton, CT). The supernatant is reserved and the pellet is rehomogenized in 3 vol of buffer A and centrifuged as before. The combined 2,600 *g* supernatants are centrifuged at 10,000 *g* (8,000 rpm) in the GSA-Sorvall rotor and the resulting supernatant is discarded. The 10,000 *g* pellets are resuspended and briefly homogenized in 0.6 M KCl, 5 mM Na-PIPES, pH 6.8, with two strokes of a motor driven Teflon/glass homogenizer followed by incubation on ice for 1 h. Salt-treated microsomes are sedimented at 90,000 *g* (32,000 rpm) in a Beckman 35 rotor (Beckman Instruments, Inc., Palo Alto, CA) and resuspended in 10% wt/wt sucrose, 0.4 M KCl, 5 mM Na-PIPES, pH 6.8. This material is layered onto discontinuous sucrose gradients (5 ml 20%, 8 ml 30%, 6 ml 35%, 5 ml 40%) containing 0.4 M KCl, 5 mM Na-PIPES, pH 6.8, and centrifuged overnight (18 h) at 26,000 rpm in a Beckman SW.27 rotor. Fractions are collected from the sucrose interfaces by aspiration with a Pasteur pipette, diluted with ice-cold glass-distilled water and pelleted at 90,000 *g* (32,000 rpm) in a Beckman 35 rotor. Pelleted membranes are suspended in 0.3 M sucrose, 0.1 M KCl, 5 mM Na-PIPES, pH 6.8, and frozen in liquid N_2 until use.

Planar Bilayer Assembly and Recording

Lipid bilayers were cast from an equimolar mixture of phosphatidylethanolamine and phosphatidylserine dissolved in decane at a concentration of 20 mg lipid/ml. Lipid solution was spread across a 300 μm diam polystyrene aperture separating two aqueous chambers designated *cis* and *trans*. The volume of each chamber was 3.0 and 3.5 ml, respectively. T tubule vesicles (10–50 μg), were added to the *cis* solution under stirring. *Cis* and *trans* solution were always the same; *cis:* 100 mM $BaCl_2$, 50 mM NaCl, 10 mM HEPES-Tris pH 7.0; *trans:* 50 mM NaCl, 10 mM HEPES-Tris, pH 7.0. All experiments were performed at room temperature. *Cis* solution was connected via an Ag/AgCl electrode and an agar/KCl bridge to the headstage input of a List L/M EPC 7 amplifier (List Electronic DA-Eberstadt, FRG). *Trans* solution was held at ground potential using the same electrode arrangement. Records were filtered at 0.1 kHz corner frequency on an 8-pole Bessel (Frequency Devices, Inc., Springfield,

MA), digitized at 1 point/ms on a 12-bit A/D converter (Kiethley Instruments, Cleveland, OH) and fed into an IBM PC/AT computer (IBM Instruments, Danbury, CT). Drugs were added to either side from stock solutions dissolved in 100% high quality methanol. Final methanol concentration in the chamber was always <1%. Control experiments showed that methanol, at concentrations used, had no effect on channel activity.

Radioligand Binding Assays

Samples of 15–40 μg protein/ml were incubated at room temperature in 1 ml of 50 mM NaCl, 10 mM Tris-HCl, pH 7.4, and the requierd concentration of (+)-[methyl-^{3}H]PN200–110 (0.05–7 nM). Specific binding was defined as the amount of radiolabel that could be displaced competitively by 1 μM cold PN200–110 or nitrendipine; [^{3}H]PN200–110 was the last reagent added. Incubation time varied from 40 to 60 min. For determination of total amount of [^{3}H]PN200–110, a small aliquot (20 μl) was removed before filtration. Binding was terminated by rapid filtration on Whatman GF/B or GF/F glass fiber filters. Filters were washed twice with 5 ml of an ice-cold solution containing 20 mM Tris-HCl pH 7.2 and 200 mM choline chloride. Nonspecific binding to filters was negligible and independent of the presence of unlabeled ligand in the incubation medium. Radioactivity was measured in 6 ml of a Beckman HP/b scintillant on a Beckman LS 3801 scintillation counter. All experiments were performed under dim light to avoid photolysis of DHPs. Binding assays in the presence of divalent cations were performed essentially as described above. Free Ca in the range of 1 to 1,000 μM was calculated using a standard computer program and was verified with a Ca electrode. In experiments using high divalent concentration, osmotic pressure was kept approximately constant by replacing calcium chloride with choline chloride. Controls showed no effect of choline chloride on [^{3}H]PN200–110 binding. Inhibition of [^{3}H]PN200–110 binding by different DHPs was carried out using 0.2 nM [^{3}H]PN200–110 and 20 μg/ml t tubule protein. Receptor occupancy by the radiolabel was 20–30%. Incubation solutions varied; solution A: 50 mM NaCl, 10 mM Tris-HCl, pH 7.2; solution B: 100 mM $BaCl_2$, 10 mM Tris-HCl, pH 7.2; C and D: same as A and B, respectively, with 1 μM racemic Bay K 8644. Additional details are given in the legend of Fig. 14. Protein concentration was determined by the Lowry method using bovine serum albumin as standard.

Chemicals

Phosphatidylethanolamine and phosphatidylserine were from Avanti Polar Lipids (Birmingham, AL). *n*-Decane was purchased from Aldrich Chemical Co. (Milwaukee, WI). (+)-[methyl-^{3}H]PN200–110 (71 Ci/mmol) was from New England Nuclear (Boston, MA). Stereoisomers with >95% purity, (−)Bay K 8644, (+)Bay K 8644, (−)nimodipine, and (+)nimodipine, were made available to us by Dr. A. M. Brown (Baylor College of Medicine, Houston, TX). The original source was Dr. Scriabine at Miles Institute, New Haven, CT. Antagonist SDZ 207–180 and PN200–100 were a gift of Dr. Ruegg at Sandoz Pharmaceutical Div., Basel, Switzerland. Racemic Bay K 8644 was a gift from Dr. Scriabine at Miles Institute, New Haven, CT. D600, D575, and D890 were made available to us by Dr. R. W. Tsien (Stanford University, Palo Alto, CA). The original source was Knoll AG (Ludwigshafen, FRG).

RESULTS

Inhibition of t Tubule Ca Channels by Verapamil Derivatives and Diltiazem

Ca channels from rat t tubules incorporated into planar bilayers are activated by *cis*-positive potentials, thus the external end of the protein must face the *trans* solu-

tion and the internal end must face the *cis* solution (Affolter and Coronado, 1985). This polarity of insertion was later confirmed pharmacologically by the use of the permanently charged phenylalkylamine, D890, a compound in which the tertiary amine of D600 is made quaternary by replacement of H with CH_3 (Affolter and Coronado, 1986). As shown in this initial study, Ca channels could be blocked when D890 was present in the *cis* solution with no effects up to a *trans* drug concentration of 50 μM. Hescheler et al., (1982) described the sidedness of D890 in ventricular

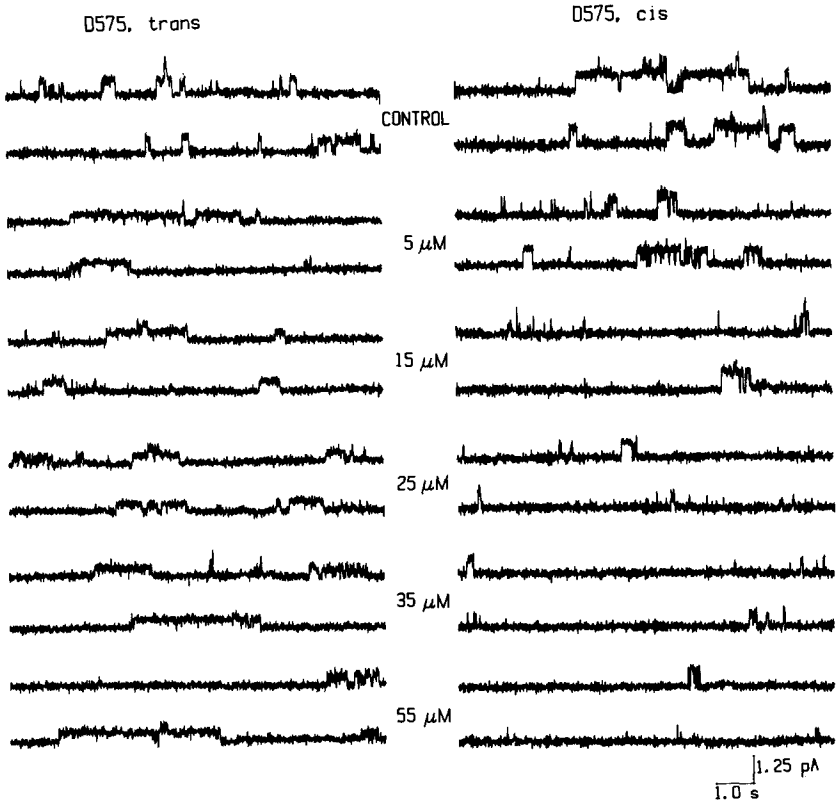

FIGURE 1. Sidedness of blockade of Ca channels by D575. Records labeled D575 *trans* and D575 *cis* are two separate experiments of Ba current at 0 mV in the presence of the quaternary phenylalkylamine D575. The fraction of time in which one or more open channels were observed, f_o, was 0.14 in *trans* control, 0.10 in *trans* 25 μM, and 0.105 in *trans* 50 μM. f_o was 0.13 in *cis* control, 0.04 in *cis* 25 μM, and 0.02 in *cis* 55 μM. Duration of each record is 10 s.

heart cells in which blocking of Ca currents was only seen after injection of the compound into cells. Evidently, the quaternary ammonium of D890 prevented the drug from freely permeating the membrane and reaching its internally located blocking site.

Fig. 1 describes a similar observation in rabbit t tubules using the quaternary derivative D575. The latter differs from D890 in the number of methoxy groups,

five in D890 (same as D600) and four in D575. Current carrier in all experiments was 100 mM $BaCl_2$ present in the *cis* solution; 50 mM NaCl was present in the *cis* and *trans* solutions. The equilibrium potential of ions are $E_{Ba} < -100$ mV (nominally minus infinity), $E_{Na} = 0$ mV, $E_{Cl} = +35$ mV. Current at 0 mV, the membrane potential of recordings of Fig. 1 and all others, is in the outward direction and is shown upwards. Control activity elicited by 1 μM racemic Bay K 8644 added to both sides is shown in top records. Approximately 90% of the fraction of time that channels spent open in control was blocked by 55 μM D575 present in the *cis* solution whereas the same *trans* concentration resulted in activity similar to that of control. Even though *trans* D575 did not block channels in an all-or-none manner, it decreased conductance by ~30%, from 0.72 pA in control (SD = 0.13 pA) to 0.51 pA (SD = 0.10 pA) at >5 μM. This decrease in conductance was specific for D575 and was not seen with D890 nor with the rest of phenylalkylamines tested. Formation of low conductance states in the t tubule Ca channel have been observed after numerous in situ biochemical interventions that alter the bilayer lipid composition, including the addition of cholesterol to the *trans* aqueous phase (Ma and Coronado, 1988*a*). Similar to the condensing effect of cholesterol in lipid bilayers (Simon et al., 1982), the *trans* effect of D575 could be mediated by disturbances in the phospholipid bilayer structure, therefore it is unrelated to occupancy of drug receptors in the Ca channel.

Dose-response curves for the two charged phenylalkylamines, D575 and D890, and the tertiary amine, verapamil, are shown in Fig. 2. From four separate experiments the ED_{50}'s for internal D890 and D575 averaged 25 and 10 μM, respectively. The lack of *trans* blockade in either case confirmed the *cis*-intracellular insertion of channels in the rabbit preparation. If the rate of passage of external drug to an internal receptor was the only factor determining the side-dependent effects of D890 and D575, these should not be present for tertiary phenylalkylamines given that the neutral form of the amine is lipid soluble (Uehara and Hume, 1985). This conclusion was supported by our results with verapamil but not those with the methoxy derivative of verapamil, D600.

Experimentally, ED_{50}'s for verapamil on either side of the channel were ~2 μM. On the other hand, the effect of D600 which has pK_a of 8.5, which is similar to verapamil (Uehara and Hume, 1985), is more peculiar and is shown in Fig. 3. *Trans* blockade was monotonic, but *cis* D600 activated channels at low concentration (<10 μM) and inhibited activity at a much higher concentration (>30 μM). This asymmetry in the action of D600 is not explained by hydrophobic partitioning and suggests that the loci of action of D600 on the *cis* and *trans* sides may be entirely different. There is evidence to suggest that activation or inhibition by phenylalkylamines depends on regulatory components other than the DHP receptor. In neuronal Ca channels, D600 and other Ca antagonists behave as agonists after activation of G proteins presumably coupled to the Ca channel (Scott and Dolphin, 1987). In planar bilayers, G_s has been shown to open t tubule Ca channels directly when added to the *cis* chamber (Yatani et al., 1988). Thus it is plausible that *cis* D600 mimics in some way the action of an endogenous t tubule G protein (Scherer et al., 1987).

Our observation differs from that made in neurons, however, in several aspects. In neurons, (*a*) activation of currents by D600 decays within minutes but is perma-

nent in our case for up to 30 min, (*b*) D600 is applied externally but only internal drug potentiates in our case, (*c*) the DHP nifedipine also potentiates currents but not in our case (see Fig. 13), and (*d*) potentiation was observed after activation of currents with internally applied GTP-γ-S; in our case, the only agonist present was the DHP Bay K 8644.

Another extreme case of asymmetric blockade by a tertiary amine was found

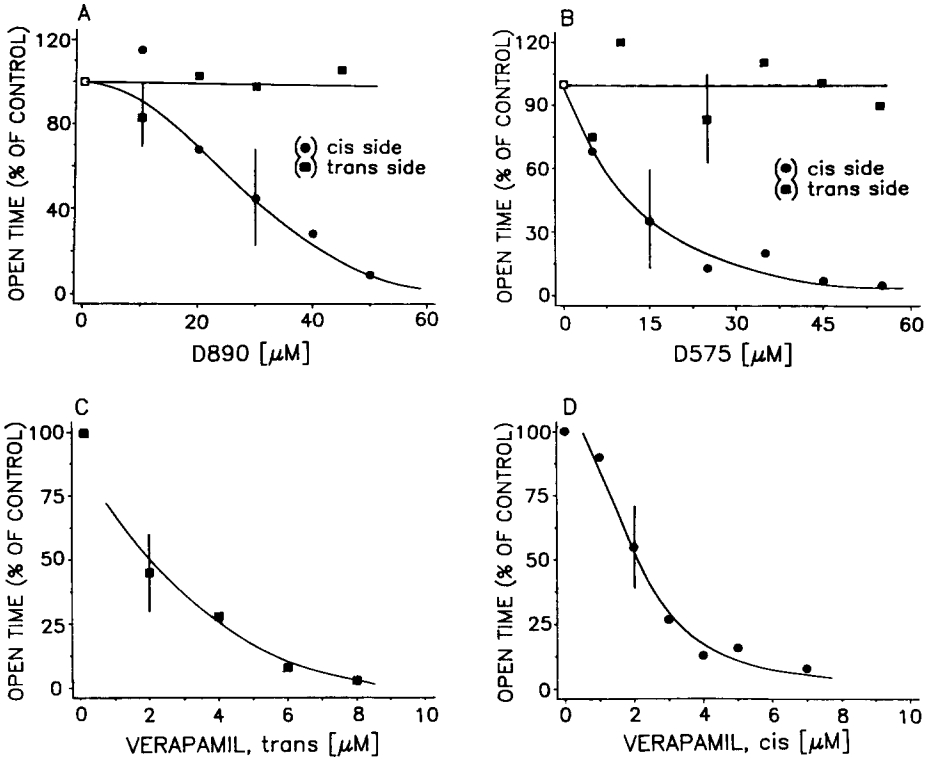

FIGURE 2. Dose-response curves of phenylalkylamines on *cis* and *trans* sides of the Ca channel. Dose-response curves at 0 mV are shown for the quaternary amines, D890, D575, and verapamil. Open time 100% corresponds to the fraction of time with one or more open channels averaged over 144 s prior to drug addition. *Cis* and *trans* titrations are from separate experiments. Bars correspond to the data point with the largest SD computed from three separate recordings.

using the benzothiazepine *d-cis*-diltiazem and is shown in Fig. 4. Over the range tested (1–100 μM) diltiazem was purely inhibitory with an internal ED_{50} of 2 μM and an external ED_{50} of 32 μM or 10-fold higher. We consider this result to be significant because ~30% of diltiazem is neutral, thus lipid-soluble at pH 7.2 (pK_a = 7.7). The opposite actions of D600 along with the sidedness of diltiazem reinforced our view that different drug receptors for the same compound may be present on the t tubule Ca channel.

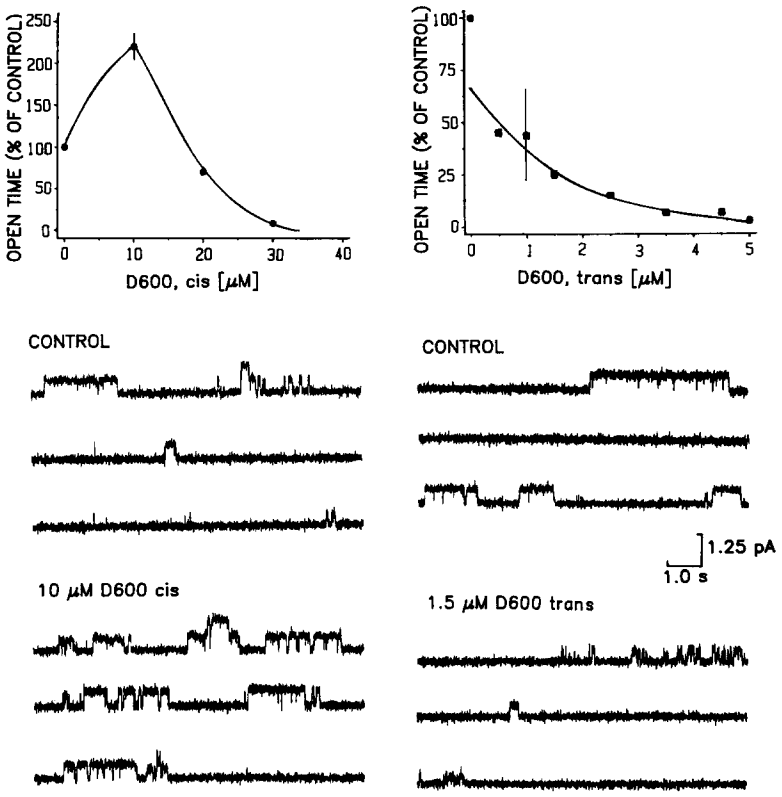

FIGURE 3. Activation and inhibition of Ca channels by D600. Channel recordings and dose-response curve for D600, a methoxy derivative of verapamil. f_o was 0.14 and 0.30 in control and after *cis* 10 μM D600, respectively. In *trans* addition, f_o was 0.16 in control and 0.04 after 1.5 μM D600. Bars correspond to two SDs of three separate recordings.

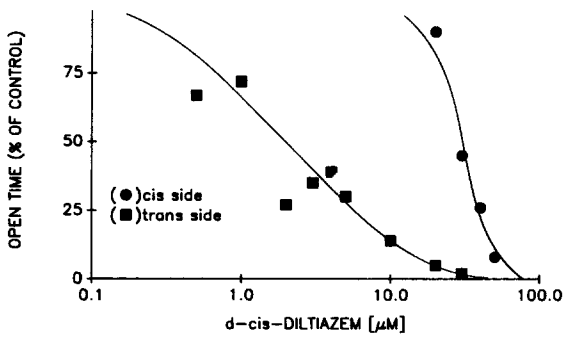

FIGURE 4. Inhibition of Ca channels by *cis* and *trans* *d-cis*-diltiazem. Dose-response curves at 0 mV were obtained separately for *cis* and *trans* additions of diltiazem. In both cases channels were activated by 1 μM Bay K 8644. Each point represents a fraction of open time averaged over 144 s at the indicated concentration of diltiazem.

Activation and Inhibition by (−)Bay K 8644

To test whether internal and external drug sites existed in the t tubule Ca channel it was essential to use pure stereoisomers since most Ca drugs are optically active, drug receptors in the Ca channel are highly stereospecific, and in some cases, the two stereoisomers of a given compound have opposite electrophysiological effects (Garcia et al., 1984; Franckowiak et al., 1985; Williams et al., 1985; Ehrlich et al., 1986; Striessnig et al., 1986; Hamilton et al., 1987; Kokubun et al., 1987). We simplified the problem by studying in detail a single compound, the agonist DHP (−)Bay K 8644 (Franckowiak et al., 1985; Lacerda and Brown, 1988). This is a chemically pure stereoisomer for which dose-response curves on either side of the channel could be measured in the absence of other compounds.

When racemic Bay K 8644 is used in planar bilayer recordings of skeletal Ca channel, micromolar levels are usually required to induce openings (Affolter and Coronado, 1985). With the pure agonist enantiomer (−)Bay K 8644, we found that openings could be elicited with nanomolar concentrations. Fig. 5 shows representative traces at 0 mV of Ca channels activated with increasing concentrations of (−)Bay K 8644 added to the external (*trans*) solution. All traces are from a single experiment (out of three) in which t tubules were added to the *cis* solution and agonist to the *trans* solution without breakdown of the film throughout the entire recording. This ensured that the agonist was in contact with the *trans* face of the inserted channel only. Unlike in rat (Affolter and Coronado, 1985), in the rabbit preparation we found no measurable activity in the absence of agonist. Threshold for activation was ~10 nM (−)Bay K 8644. At this concentration we did not observe the long open events characteristic of channels activated by racemic Bay K 8644 described in heart (Hess et al., 1984), and by us in skeletal muscle (Affolter and Coronado, 1985). At 25 nM and above, frequency of openings, channel lifetime, and fraction of time spent open increased with concentration, more in line with results using the racemic compound.

As shown in Fig. 6, in the range of 10 to 250 nM (−)Bay K 8644 there is an approximately linear increase in the fraction of time in which one or more channels were open. On the other hand, the frequency of openings or number of events per unit time, had a plateau at concentrations above 100 nM and decreased considerably at 250 nM (−)Bay K 8644. By comparing the left and right panels of Fig. 6 it is clear that above 100 nM (−)Bay K 8644 the fraction of time in which one or more channels were open increases at the expense of longer openings since the frequency of events decreases considerably at high drug concentration. Over the range tested we observed no changes in channel conductance of the kind reported by Lacerda and Brown (1988) in heart cells or of the kind seen in *cis* additions of (−)Bay K 8644 (see Fig. 8 below).

The distribution of observable lifetimes at a cutoff frequency of 100 Hz was biexponential with both time constants increasing with drug concentration. Time histograms are shown as insets in Fig. 7. Histograms were plotted in a cumulative form as percent of events lasting the specified time or longer. Each fitted exponential is also shown in the histograms. A time constant that fitted the brief events, denominated fast τ, is predominant at 10 and 25 nM, while a second component, slow τ, is more apparent at higher doses. The upper and lower panels of Fig. 7 show that fast τ and

slow τ reached a plateau at ~300 nM with limiting values of 70 and 400 ms, respectively. The midpoint (ED_{50}) for the concentration dependence of the fast τ and slow τ was 80 and 68 nM, respectively. This affinity is in the same range as the ED_{50} of (−)Bay K 8644 necessary to displace bound [^{3}H]PN200-110 in ligand binding experiments (see the legend to Fig. 14). Although lifetimes of open channels had a tendency to saturate with increasing concentration of (−)Bay K 8644, this was not

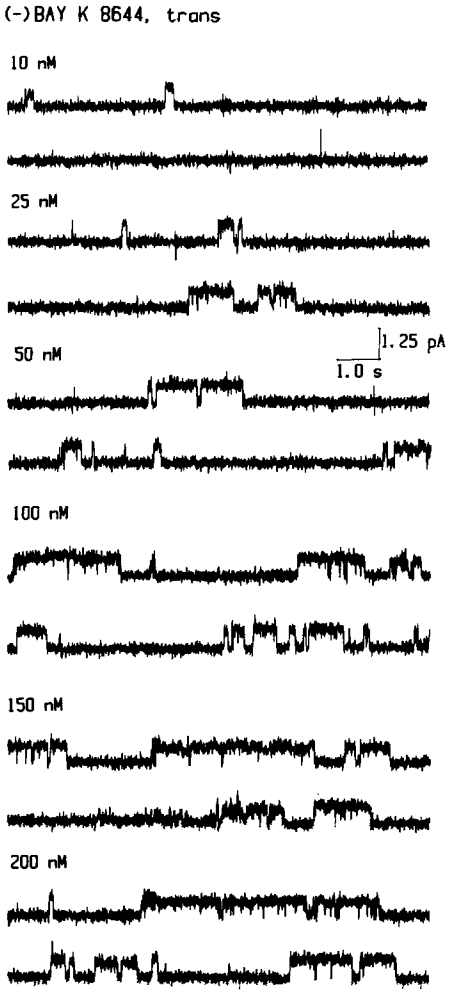

FIGURE 5. Activation of Ca channels by *trans* (−)Bay K 8644. Activity at 0 mV is shown as a function of (−)Bay K 8644 added to the *trans* side (racemic Bay K 8644 was not present). No openings were seen in the absence of the agonist. The two records at each concentration are representative of 150 s of recording time at that concentration. All data are from the same recording.

the case for the dose-response curve of open probability. On the contrary, the experimental *NP* product (*N* is the number of channels, *P* is the open probability per channel) increased with concentration over the same range in which lifetimes were actually constant (compare the left panel of Fig. 6 with Fig. 7). In separate experiments covering the micromolar range of *trans* (−)Bay K 8644, from 0.5 to 10 μM, the lifetime of channels and the *NP* product was invariant with concentration.

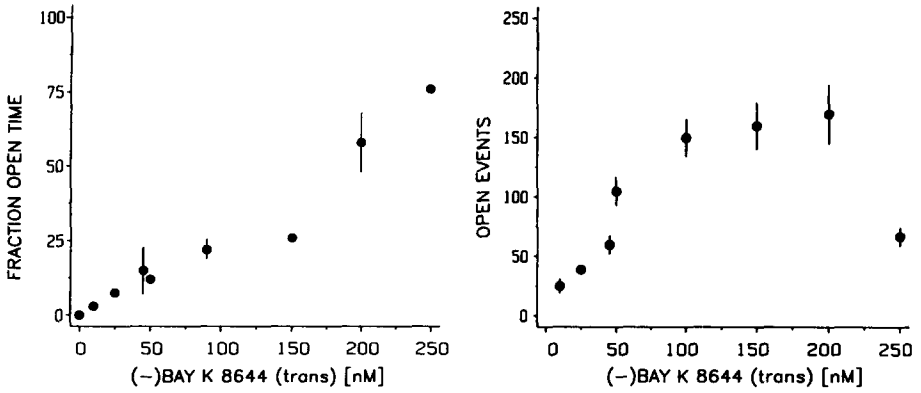

FIGURE 6. Dependence of open probability and frequency of events on (−)Bay K 8644 concentration. Fraction open time corresponds to the total time in which one or more open channels were observed relative to 144 s of total recorded time. Open events are the total number of openings during 72 s. Single points are from one recording; bars are two SDs of three separate recordings.

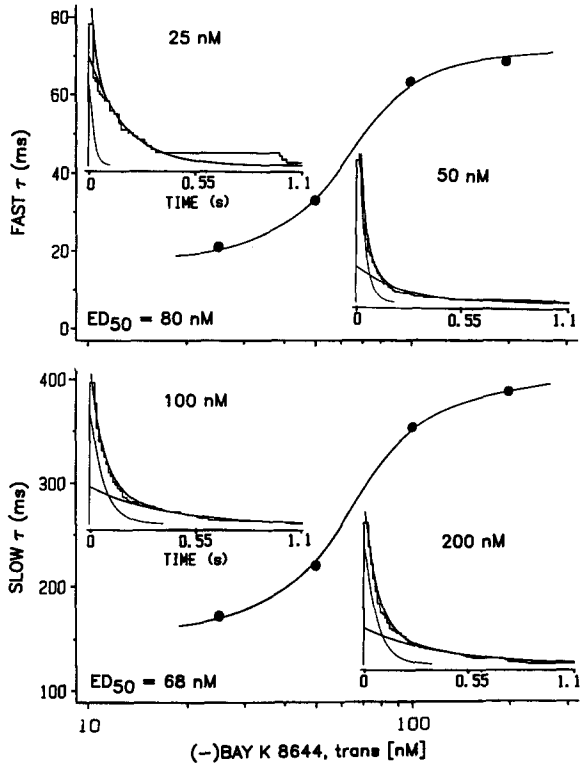

FIGURE 7. Slow and fast lifetimes of Ca channels activated by *trans* (−)Bay K 8644. Open time histograms and fitted exponentials are shown as insets at the indicated concentrations of (−)Bay K 8644. Numbers of openings were 48, 133, 162, and 191 for 25, 50, 100, and 200 nM, respectively (events <2 ms were excluded). Main curve in top and bottom represent a fit of the concentration dependence of fast τ and slow τ to a single binding site isotherm with K_d's of 80 and 68 nM, respectively.

Both parameters saturate with increasing concentration of (−)Bay K 8644 although the *NP* product does so at concentrations much higher than those measured in radioligand binding experiments.

When (−)Bay K 8644 was added to the internal side, rather than to the external solution, the results were surprising. Based on the high lipid solubility of DHPs (Rhodes et al., 1985) we expect *cis* effects to be qualitatively similar to those seen on the *trans* side. Instead, *cis* (−)Bay K 8644 did not activate channels until the concentration reached the micromolar range (0.5 μM). At low nanomolar concentrations

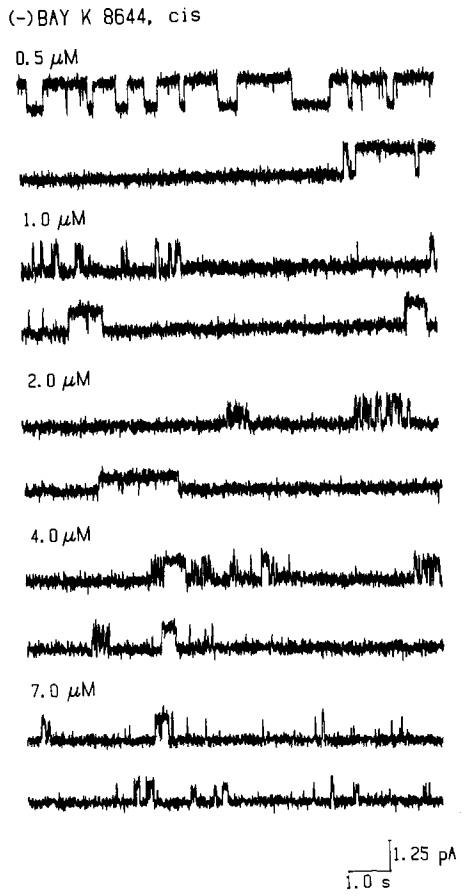

FIGURE 8. *Cis* activation of Ca channels by (−)Bay K 8644. Channel currents in Ba at 0 mV and the indicated concentrations of (−)Bay K 8644 added to the *cis* side (racemic Bay K 8644 was not present). f_o at 0.5 μM *cis* (−)Bay K 8644, the lowest concentration at which channels were observed, was 0.28. f_o decreased to 0.10 at *cis* 7 μM (−)Bay K 8644. Mean channel conductance was 1.05, 0.85, 0.87, 0.75, and 0.88 pA at 0.5, 1.0, 2.0, 4.0, and 7.0 μM, respectively (largest SD was 0.15 pA). Records are from a single experiment.

we failed to record openings with a stable frequency even though a few sporadic events were observed in some experiments. Furthermore, Fig. 8 shows that concentrations above this threshold decreased mean duration, which is the opposite of the result seen with *trans* (−)Bay K 8644. At *cis* 0.5 μM (−)Bay K 8644 there is also a significant increase in mean amplitude of channels from 0.72 pA (SD = 0.1 pA, n = 1,590) for *trans* activation, to 0.99 pA (SD = 0.15, n = 210) for the *cis* activation. This difference translates in an increase in slope conductance from ~12 to 17 pS at 0 mV. Amplitude differences induced by *cis* or *trans* agonist were less notice-

able at *cis* concentrations above 5 μM (−)Bay K 8644. Time histograms of collected openings from two separate recordings at four *cis* concentrations are shown as insets in Fig. 9. Fast and slow time constants decrease from 35 and 400 ms at 0.5 μM to 22 and 80 ms at 250 nM, respectively. Evidently, (−)Bay K 8644 added to the internal and external solution appears to activate channels by reaching into functionally different DHP receptor sites.

Sidedness of Inhibition of PN200-110 and Other DHPs

The significant difference between *cis* and *trans* activation by agonist (−)Bay K 8644 prompted the question of whether the same sidedness was present for antagonist

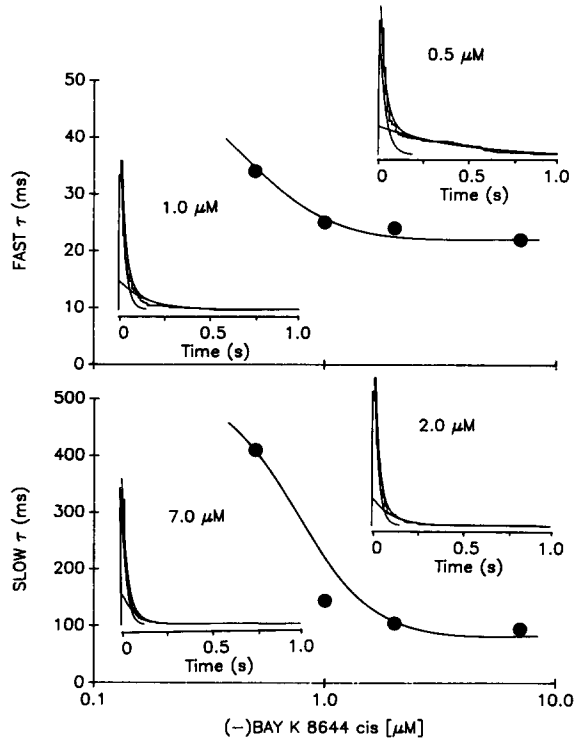

FIGURE 9. Slow and fast lifetimes of Ca channels activated by *cis* (−)Bay K 8644. Data from two separate recordings were pooled and used to fit time constants. Histograms are shown as insets at the indicated concentrations of (−)Bay K 8644. Number of openings were 210, 189, 178, and 194 for 0.5, 1.0, 2.0, and 7.0 μM, respectively (events <2 ms were excluded).

DHPs. Fig. 10 shows *trans* inhibition by the high affinity antagonist PN200-110 while a comparison of *cis* and *trans* effects is shown in Fig. 11. In these and subsequent experiments, channels were activated by 1 μM racemic Bay K 8644 present in both sides. This concentration was sufficient to activate channels internally (Fig. 7) and externally (Fig. 8). Control activity therefore represented a state in which both "internal" and "external" drug receptors were occupied by agonist. Activity was measured as the fraction of time that one or more channels spent open averaged over 144 s of recording time. As shown in records of Fig. 10, a *trans* concentration of 10 nM PN200-110 was sufficient to induce blocked times that lasted 5–10 s, which were not seen in control. Almost complete inhibition of opening events

occurred at 100 nM or higher concentrations. Qualitatively, the same kinetic sequence seen in activation by *trans* (−)Bay K 8644 is reverted by *trans* PN200-110. That is, long openings are blocked at low antagonist concentration while short openings are blocked at high concentration. Fig. 11 shows that *cis*-added PN200-110 (150 nM) had virtually no effect while the lower *trans* concentration (100 nM) blocked most openings.

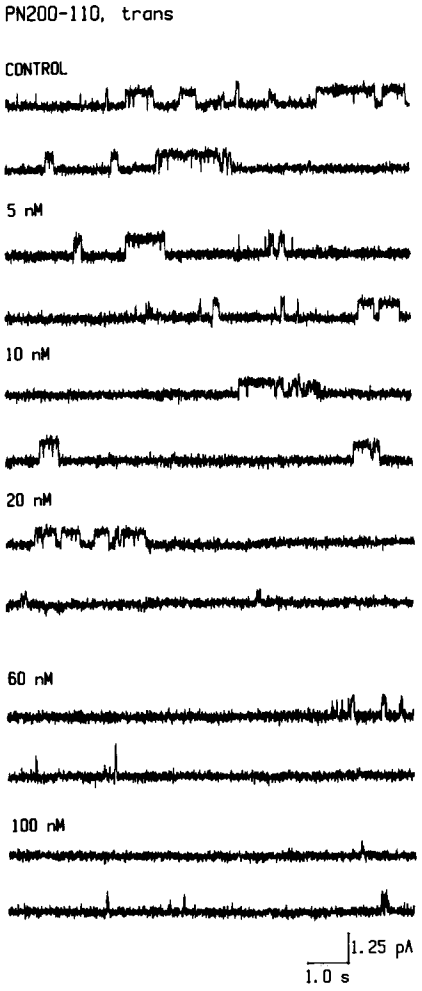

FIGURE 10. *Trans* inhibition of Ca channels by PN200-110. Ba currents were recorded at 0 mV in the presence of 1 μM racemic Bay K 8644. The antagonist PN200-110 was added to the *trans* side only. f_o for control averaged 0.24 and decreased to 0.01 at 100 nM PN200-110.

Dose-response curves constructed separately for *cis* and *trans* additions of DHPs are shown in Fig. 12 for PN200-110, nitrendipine, (−)nimodipine, and (+)Bay K 8644. Each point in each dose-response curve represents 144 s of monitored activity. For all DHPs tested the apparent affinity of inhibition was higher when drugs were added to the *trans* solution as compared with the *cis* solution. In the most extreme case, that of PN200-110, there is an almost parallel shift of the dose-response curve by 1.2 orders of magnitude towards lower affinity when the DHP is

added internally (*trans* 10 nM vs. *cis* 168 nM). In the case of nitrendipine, (−)nimodipine, or (+)Bay K 8644, the shift is less, ~0.3–0.4 orders of magnitude. Sidedness was observed with various degrees in all tested DHPs except in the case of the quaternary DHP S207-180 (*cis* and *trans* ED_{50}'s for all DHPs tested are given in the legend to Fig. 15). Except for nitrendipine, all *trans* ED_{50}'s are in the submicromolar range with PN200-100 displaying the highest affinity.

Neither *cis* nor *trans* affinities for DHPs changed when Bay K 8644 was separately present on the *cis* or *trans* sides of the channel. This is shown in Fig. 13 where the four dose-response curves are for nifedipine added either to the *cis* (*circles*) or the *trans* (*squares*) sides with racemic Bay K 8644 present in the *cis* (*filled symbols*) or *trans* (*open symbols*) solution. Each curve corresponds to a separate experiment in which the planar bilayer never broke during the recording and there was no mixing

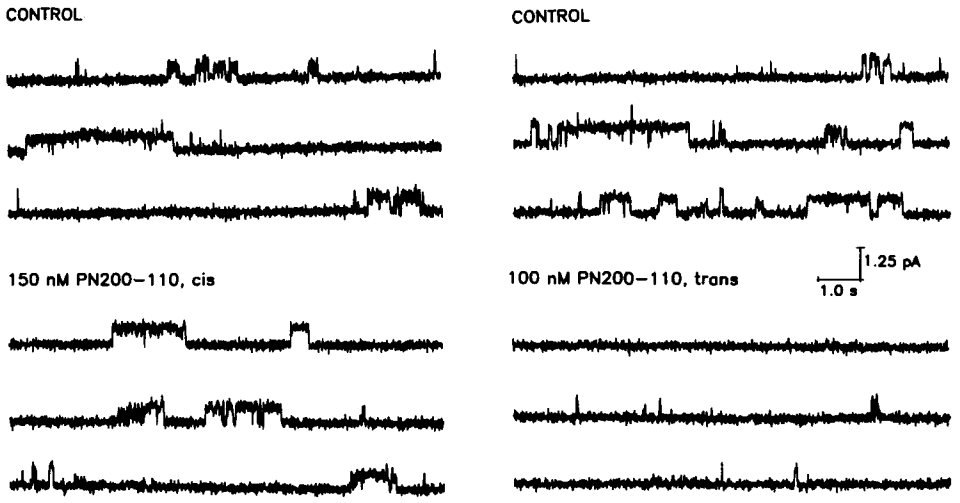

FIGURE 11. *Cis* and *trans* PN200-110 blockade of Ca channels. Single-channel recordings are for Ba current at 0 mV. *Cis* and *trans* additions are from separate recordings. f_o was 0.14 before and 0.12 after *cis* addition of 150 nM PN200-110. In *trans* addition f_o was 0.16 in control and 0.0048 after 100 nM PN200-110.

of *cis* and *trans* solutions. Curves for *cis* and *trans* inhibition are essentially independent of the presence of agonist in the same or opposite solution. The observed sidedness of DHP antagonist is therefore independent on the occupancy of the channel by Bay K 8644.

Comparison of Ca Channel and DHP Receptor Affinities for DHPs

To compare the *cis* and *trans* ED_{50}'s of channels with ligand binding affinities of DHPs we first studied in Fig. 14 the effect of $BaCl_2$ and Bay K 8644 on binding of [^{3}H]PN200-110 and its inhibition by unlabeled PN200-110 (*top right*), nimodipine (*top left*), nifedipine (*bottom left*), and nitrendipine (*bottom right*). Similar curves were made for all DHPs tested in single-channel experiments (see legend to Fig. 14). Barium ions and Bay K 8644 were present at the same concentrations used in single-

channel recordings. We avoided the use of [^{3}H]Bay K 8644 as radioligand for technical reasons. In cardiac (Janis et al., 1984*a*) as well in skeletal muscle (Valdivia H., and R. Coronado, unpublished observations) bound [^{3}H]Bay K 8644 shows a biphasic dissociation curve when displaced by other DHPs. At the concentration used for electrophysiological experiments (1 μM), the exact relative occupancy of high and low affinity sites by Bay K 8644 is difficult to measure and any interpretation of the relative potency of antagonists to displace [^{3}H]Bay K 8644 from either

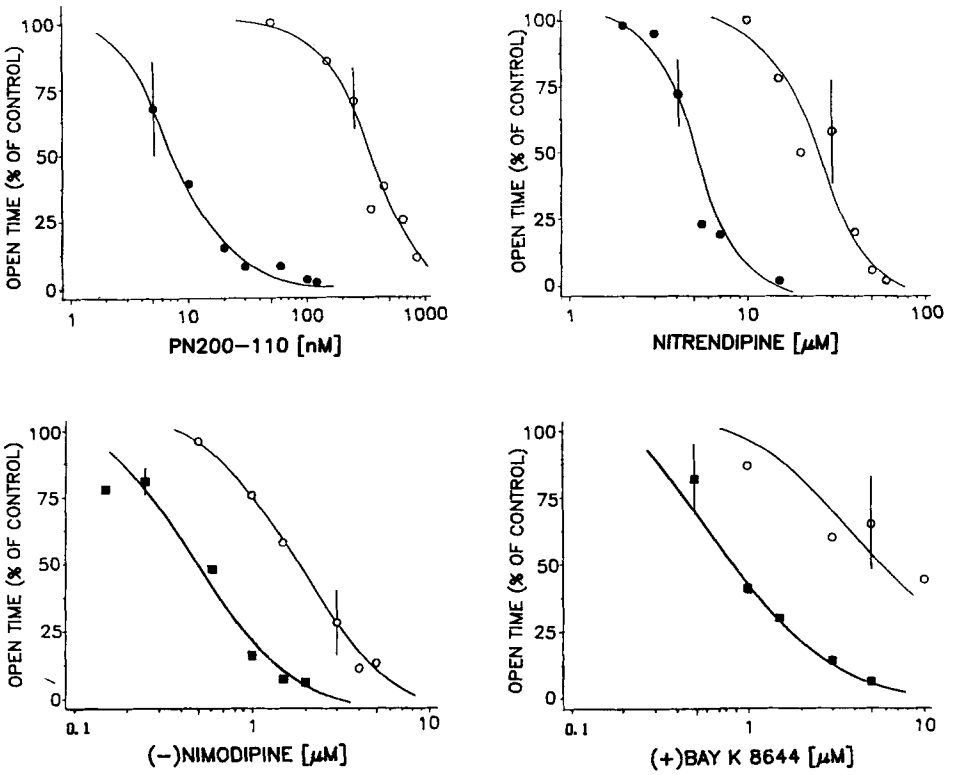

FIGURE 12. Dose response of DHPs on *cis* and *trans* sides of the Ca channel. Dose-response curves at 0 mV are shown for the antagonist DHPs PN200-110, nitrendipine, (–)nimodipine, and (+)Bay K 8644. f_o was measured during 150 s at each concentration. *Cis* and *trans* titrations were performed on different experiments. f_o control was measured during 180–300 s before drug addition and was used to normalized data. Bars represent two standard deviations of data points with the largest errors. Each point is the average of three separate titrations.

site is complex. Also, even at nanomolar concentration, [^{3}H]Bay K 8644 displays a high nonspecific binding which accounts for the poor signal-to-noise ratio reported for this compound (Janis et al., 1984*a*; Rampe et al., 1989). We used instead (+)[^{3}H]PN200-110, which is a high affinity, low background DHP that (*a*) binds to a single class of sites (Janis et al., 1985), (*b*) has been shown to track the Bay K 8644 binding site in competition experiments (Rampe et al., 1989), and (*c*) its radioligand

binding properties has been extensively characterized in skeletal muscle transverse tubules (Fosset et al., 1983; Borsotto et al., 1984). In 50 mM NaCl and contaminant levels of divalents, a Scatchard analysis of [^{3}H]PN200-110 binding to our preparation of t tubules revealed an average density of binding sites (B_{max}) of 40 pmol/mg and K_d of 0.78 nM (not shown) which is in excellent agreement with the binding capacity of DHPs reported by Fosset et al. (1983) and the binding affinities of Borsotto et al. (1984) in the same preparation.

The top left of Fig. 14 shows specific binding of [^{3}H]PN200-110 and displacement by unlabeled PN200-110 in 50 nM NaCl (*open circles*). The ED_{50} of the displacement was 1 nM PN200-110 and the apparent Hill coefficient was 1.03. Binding parameters were obtained from least-squares regression of data from three t tubule preparations. All curves in Fig. 14 were scaled using the specific binding of 0.2 nM [^{3}H]PN200-110 as control. At that drug and receptor concentration, receptor occupancy was no more than 25%. Hence, ED_{50}'s for inhibition of [^{3}H]PN200-110 binding closely follow the apparent affinity of the inhibitor DHP for the DHP

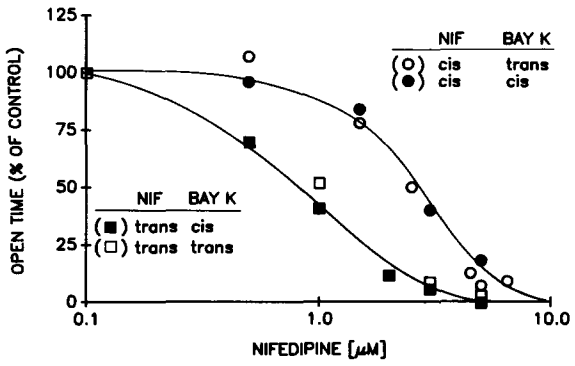

FIGURE 13. Inhibition of Ca channels by nifedipine in the presence of *cis* or *trans* Bay K 8644. Dose-response curves at 0 mV were obtained in four separate experiments for each of the following conditions. *Filled symbols:* Ca channels activated by 1 μM Bay K 8644 added to the *cis* side with nifedipine added to the *cis* (*circles*) or *trans* (*squares*) side. *Empty symbols:* Ca channels activated by 1 μM Bay K 8644 added to the *trans* side and nifedipine added to the *cis* (*circles*) or *trans* (*squares*) side. Points are the fraction of time that one or more channels spent open averaged over 144 s and normalized relative to activity in the absence of nifedipine.

receptor (see Cheng and Prusoff, 1973). When the displacement by unlabeled PN200-110 is done in the presence of 1 μM racemic Bay K 8644 (Fig. 14, *open squares*), the inhibition curve was displaced to the right with an apparent K_d of 10 nM and an apparent Hill coefficient 1.05. Similar displacements were present for all DHPs tested (compare open circles and open squares in Fig. 14). The absolute specific binding in the presence of 1 μM Bay K 8644 was ~30% of that seen in the absence of the agonist since Bay K 8644 itself displaces PN200-110 with an ED_{50} of ~100 nM (not shown).

The effect of 0.1 M $BaCl_2$ on binding parameters of PN200-110 are shown in Fig. 14 (*top left*) in the absence (*filled circles*) or presence (*filled squares*) of 1 μM Bay K 8644. An increasing concentration of $BaCl_2$ in the millimolar range inhibited PN200-100 binding, which is again in agreement with the results of Fosset et al., (1983) and Galizzi et al., (1984) in the same preparation. In our study, 0.1 M Ba decreased the B_{max} of PN200-110 binding by ~60% without a major change in affinity (average B_{max} of 40 pmol/mg, K_d of 0.78 nM in 50 mM NaCl; and average B_{max} =

16 pmol/mg, K_d = 0.92 nM, in 50 mM NaCl plus 0.1 M $BaCl_2$; data not shown). In addition to decreasing the number of available sites, Fig. 14 shows that barium induced an unexpected change in the apparent Hill coefficient of the displacement reaction, from close to unity in 50 mM Na (*open circles*) to 0.4–0.6 in 50 mM Na plus 100 mM Ba (*filled circles*). The change in steepness of inhibition induced by barium

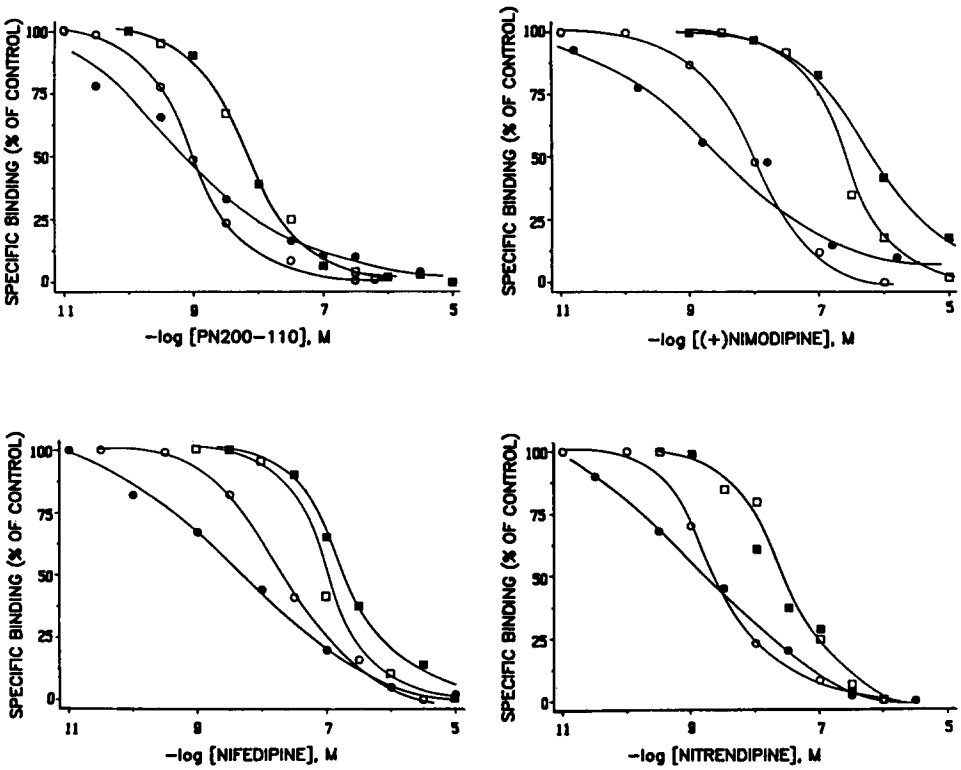

FIGURE 14. Displacement of PN200-110 binding by DHPs under various conditions. [^{3}H]PN200-110 (0.2 nM) was incubated with 10–30 μg rabbit t tubule vesicles and the indicated concentrations of racemic PN200-110, (+)-nimodipine, nifedipine, and nitrendipine. Incubation solutions were, solution A: 50 mM NaCl, 10 mM HEPES-Tris pH 7.2 (*empty circles*); solution B: 100 mM $BaCl_2$, 50 mM NaCl, 10 mM HEPES-Tris pH 7.2 (*filled circles*); solution C: solution A plus 1 μM Bay K 8644 (*empty squares*); solution D: solution B plus 1 μM Bay K 8644 (*filled squares*). Specific binding was defined as counts per minute retained in the absence of displacer minus counts per minute retained in the presence of 1 μM PN200-110. This number was normalized to 100% and, in absolute terms, was ~3,000 cpm for solution A and 2,000 cpm for solution B. In experiments using Bay K 8644 (solutions C and D) only a third of these values were retained. Signal-to-noise ratio when using solutions C and D was increased by doubling the amount of protein and volume of incubation so that free [^{3}H]PN200-110 was kept constant. ED_{50}'s in units of -log[DHP] for displacement of bound [^{3}H]PN200-110 in the absence and presence of 1 μM Bay K 8644 were, respectively, (−)Bay K 8644: 7.0, 6.3; (+)Bay K 8644: 8.2, 6.6; nimodipine: 8.0, 6.5; (−)nimodipine: 8.4, 7.4; (+)nimodipine: 8.1, 6.5; nitrendipine: 8.7, 7.4; S-207-180: 7.4, 6.2; nifedipine: 8.3, 7.0; PN200-110: 9.0, 8.0. All SDs were within 0.2 log units of reported mean.

can be clearly seen in each of the four displacement curves in Fig. 14 and, in fact, it was present for all DHPs. Even more peculiar is the fact that no change in Hill coefficient was observed when displacement was done in the presence of 1 μM Bay K 8644 (compare open and filled squares in Fig. 14). We interpret this change in steepness as a manifestation of two interacting DHP sites one of which is either unmasked or induced by high barium. Direct electrophysiological evidence favoring two DHP sites was found by comparing *cis* and *trans* ED_{50}'s with radioligand binding ED_{50}'s for all DHPs tested.

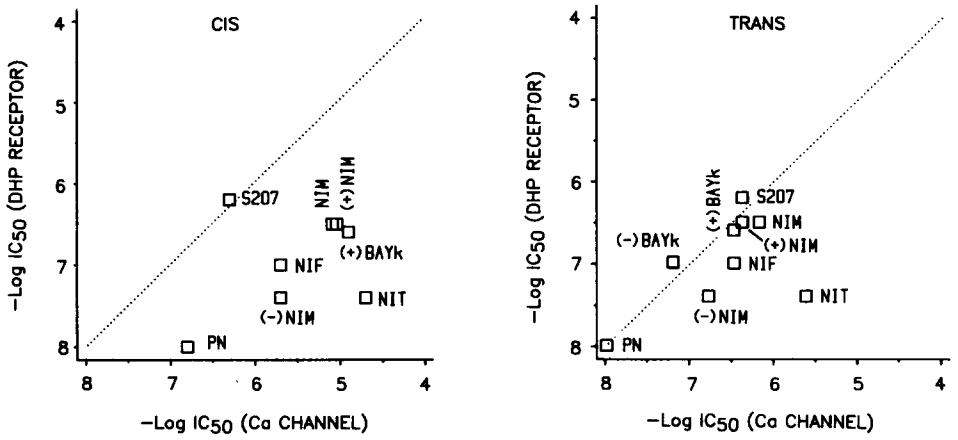

FIGURE 15. Correlation between ligand binding affinities and DHP blockade of Ca channels. (*Left panel*) Ligand binding ED_{50}'s of DHPs obtained by displacement of [^{3}H]PN200-110 (y-axis) are compared with single-channel ED_{50}'s obtained by inhibition of fraction open time using *cis*-added DHPs (x-axis). In both sets, experiments were performed at 0 mV in the presence of 1 μM racemic Bay K 8644, 0.1 M Ba. Dotted line has a slope equal to 1. (*Right panel*) Ligand binding ED_{50}'s are compared with ED_{50}'s for activation [(−)Bay K 8644)] and inhibition (all other DHPs) of fraction open time using *trans*-added DHPs (x-axis). As channel activation by (−)Bay K 8644 did not require the presence of racemic Bay K 8644, its single-channel ED_{50} was compared with ligand binding ED_{50} in the absence of racemic agonist. The dotted line has a slope equal to 1. Channel ED_{50}'s in -log[DHP] units were for *cis* and *trans*, respectively: PN200-110 (*PN*): 6.8, 8.0; nifedipine (*NIF*): 5.7, 6.5; S207-180 (*S207*): 6.3, 6.4; nitrendipine (*NIT*): 4.7, 5.7; (+)nimodipine [(+)*NIM*]: 5.0, 6.4; (−)nimodipine [(−)*NIM*]: 5.7, 6.8; nimodipine (*NIM*): 5.1, 6.2; (+)Bay K 8644 [(+)*BAYk*]: 4.9, 6.5; (−)Bay K 8644 [(−)*BAYk*]:—7.2. DHP receptor IC_{50}'s in both graphs were PN200: 8.0; nifedipine: 7.0; S207-180: 6.2; nitrendipine: 7.4; (+)nimodipine: 6.5; (−)nimodipine: 7.4; nimodipine: 6.5; (+)Bay K 8644: 6.6; (−) Bay K 8644: 6.3. All SDs were within 0.2 log units of the reported mean.

Correlation of ED_{50}'s in single-channel and binding experiments are shown in Fig. 15. *Cis* addition of DHPs (*left panel*) and *trans* addition of DHPs (*right panel*) were plotted separately while binding data is the same for both figures. The dotted line in both graphs has a slope of unity. All x, y entries are the same given in the figure legend. The slope (n) and correlation coefficient (r) for the set of x, y pairs was n = 0.45, r = 0.85 in the left panel and n = 0.8, r = 0.87 in the right panel. When nitrendipine data is omitted, because it shows a notorious lack of correlation in both

cases, the slope and correlation coefficient became $n = 0.55$, $r = 0.89$ (*left panel*) and $n = 0.9$, $r = 0.92$ (*right panel*).

As a whole we conclude that the correlation holds quantitatively for *trans* additions or DHPs but not for *cis* additions. Surprisingly, the only DHP that shows equal effects on both sides and has a 1:1 correspondence in binding and channel experiments is the permanently charged DHP S207-180. In -log units S207-108 shows ED_{50}'s 6.3, 6.4, and 6.2 for *cis*, *trans*, and binding affinities, respectively. In fact, S207-180 is the only DHP that in *cis*-addition experiments is close to the dotted line. All other DHPs in *cis*-addition experiments are below the dotted line implying that channel affinities are lower than binding affinities. In the most severe cases, there is an approximate 50-fold lower channel affinity for *cis*-added (+)Bay K 8644 and (−)nimodipine and a 500-fold lower channel affinity for *cis*-added nitrendipine. In contrast, *trans*-added DHPs and binding data shows a remarkable agreement, with the sole exception of nitrendipine. For *trans* nitrendipine the discrepancy is 50-fold, not 500-fold as for *cis*-added drug.

DISCUSSION

In the present report we outlined the pharmacology of the Ca channel of skeletal muscle incorporated into planar bilayers and correlated ED_{50}'s for single channels and DHP receptors. Given the open chamber arrangement of planar bilayers we were naturally inclined to test drug effects separately on the cytosolic and extracellular sides. Significant differences in internal and external affinities were found for the three classes of Ca antagonists (namely DHPs, phenylalkylamines, and diltiazem) although only the DHPs were studied with sufficient detail. The pharmacokinetic interactions among these drugs, inferred from radioligand binding experiments, is complex. In skeletal muscle, DHPs bind with nanomolar affinity to an apparently single class of sites in the α_1 peptide (reviewed by Campbell et al., 1988; Catterall et al., 1988; Hosey and Lazdunsky, 1988). Binding of DHPs is enhanced by benzothiazepines such as diltiazem and inhibited by phenylalkylamines such as verapamil via drug occupancy of a separate set of receptors allosterically coupled to the DHP receptor. Photoreactive derivatives of the three classes of drugs compete for covalent binding to the α_1 peptide of the DHP receptor (Galizzi et al., 1986; Stiessnig et al., 1986; Siber et al., 1987), which suggests that the same protein contains the three receptor sites for calcium antagonists. Our results differ from this view in that DHPs act on t tubule Ca channels as if they were bound to more than one site. Inasmuch as constant channel activity at 0 mV was induced in all cases by agonist DHP Bay K 8644, we investigated in detail whether the sidedness of DHP blockers varied with the presence of *cis* or *trans* agonist. The ED_{50}'s for nifedipine shown in Fig. 13 indicated that this was not the case. This result rules out the most trivial explanation of sidedness, which is that the agonists could have interfered with the partitioning of the antagonists in the lipid phase when both were present in the same solution. Instead, sided-dependent effects appeared to be an intrinsic characteristic of the Ca channel protein since they were present even when (−)Bay K 8644, the pure stereoisomer agonist (Francowiak et al., 1985) was tested in the absence of other drugs.

Side-dependent effects of DHPs were surprising because their lipid solubilities

are extremely high and there was no previous indication that their diffusion rates in membranes were in any way restricted (Rhodes et al., 1985). Except for S207-180, we find that all DHPs have significantly lower *cis* than *trans* affinities. The latter is a permanently charged analogue of PN200-110 in which the quaternary ammonium is separated from the DHP ring by 10 methylene groups. Apparently this spacer chain is sufficiently long to ensure that the DHP moiety has no net charge, which would have otherwise reduced the pharmacological potency of the compound as in the case of a quaternary DHP tested by Uehara and Hume (1985). Not only apparent affinities are different between the inside and the outside but the ranking order of DHPs is also changed. Potencies on the *cis* sides are in the sequence PN > S207 > NIF ~ (−)NIM > (+)NIM ~ NIM > (+)BAY K > NIT, while on the *trans* side the sequence is PN > (−)NIM > NIF ~ S207 ~ (+)NIM ~ +(BAY K > NIM > NIT. In both sequences, the affinity for PN200-110 is the highest. The latter is in agreement with binding data (Fig. 14) and also in agreement with single-channel data in cardiac DHP channels (Hamilton et al., 1987). On both sides also, (−)nimodipine is more effective than (+)nimodipine and the agonist action of (−)Bay K 8644 has higher potency than that of the antagonist (+)Bay K 8644. Again, these two results are in agreement with findings in heart Ca channels (Hamilton et al., 1987). Therefore, drugs added to either solution appear to gain access to a bona fide DHP site although there are recognizable differences between *cis* and *trans* effects and only *trans* ED_{50}'s correlate well with receptor binding ED_{50}'s.

We have considered the possibility that *cis* and *trans* effects of DHPs reflect differences in accessibility of DHPs to a single class of receptor sites in equilibrium with the external bulk solution. In such a case, *cis*-added DHPs would have to negotiate energetically its passage across the membrane to reach the binding site. The lower *cis* affinity could then be explained by a combination of factors including differences in partition coefficients, ligand asymmetry, lateral diffusion, interface distribution, and in general, by parameters that determine the diffusion rate of a solute across a membrane. We consider this alternative unlikely because, in our hands, the onset of DHPs was almost instantaneous in all cases and did not change significantly with time. That is, ED_{50}'s did not depend to any great extent on the time of exposure of channels to DHPs as would be the case if access rates were the limiting factor. Moreover, chemically identical DHPs, such as pairs of enantiomers, appear to have different stereoselectivities on both sides. For example, (−)nimodipine has a 25-fold difference between *trans* and *cis* ED_{50}'s while (+)nimodipine has a difference of only 12-fold. The ratio of affinities (+)nimodipine/(−)nimodipine is 2.5 on the *trans* side and 4.8 on the *cis* side. Because both steroisomers are expected to have identical diffusional properties it is unlikely the *cis* and *trans* differences reflect a difference in the rate of drug passage across the membrane.

Rather than an accessibility difference, we favor the hypothesis that the sidedness of DHPs reflects the presence of a second DHP site associated with the Ca channel. Besides the results described for nimodipine above, there are significant differences in *cis* and *trans* effects of (−)Bay K 8644 which suggest two chemically different DHP binding sites. As shown in Fig. 5 the pure enantiomer agonist increased channel lifetime on the *trans* side but when present in the *cis* side it decreased lifetime (Fig. 8). Further ligand binding evidence for two sites in this study is provided by the

decrease in apparent Hill coefficient or negative cooperativity seen in the presence of high Ba (Fig. 14). The existence of high and low affinity DHP sites have been considered in numerous studies. Janis et al. (1984*a*) suggested this possibility on the basis of a change in Hill coefficient for Bay K 8644 binding to heart membranes. The same conclusion was reached by others, based on a differential effect of diltiazem on binding of DHP agonist and antagonists to cardiac membranes (Maan and Hosey, 1987; Rampe et al., 1989). Hamilton et al. (1987) and Williams et al. (1985) suggested two interacting sites in the Ca channel on the basis of the voltage dependence of DHPs, while Kokubun et al. (1987) noticed two sites in studies with pure enantiomer agonist-antagonist pairs of the DHP 202-791. An independent confirmation of at least two binding sites in the DHP receptor of skeletal muscle has been provided by Scatchard analysis of PN200-110 binding. At 100 mM Ba there is a reduced binding capacity of [^{3}H]PN200-100 to essentially a single class of binding sites (Fig. 14). At low Ba (10 mM), the Scatchard relationship is nonlinear with at least two binding components (Valdivia H., and R. Coronado, unpublished observations). Interestingly, curvilinear Scatchard plots for PN200-110 binding were not seen in Ca (Fosset et al., 1983) which is the divalent of choice in most binding studies but not in single-channel recording. Thus radioligand experiments as well as cellular recordings have recognized the need to postulate several DHP receptors associated to the Ca channel.

The correlation of receptor binding and single-channel effects of DHPs suggested strongly that the pure component of the Ca channel and the 170 kDa α_1 DHP receptor peptide are closely related and probably physically coupled. This conclusion stems from our finding that when all sources of discrepancies between DHP receptor binding and Ca channel measurements are removed, the agreement between DHP effects on radioligand binding and *trans* channel inhibition is outstanding. Only one compound, nitrendipine, has a grossly low affinity in single-channel experiments and at the present we have no clues as to why this is the case. However, we realize that only one of three blocking mechanisms applicable to our protocols would predict a perfect 1:1 correlation between DHP blocking and binding experiments. Since the channel did not open without agonist (Fig. 5), an open channel is a channel with agonist bound to it. Agonist-bound open channels may be blocked by an antagonist by one of three modes: (*a*) by displacement of the agonist and binding to the same site i.e., a competitive mode; (*b*) by binding to a separate site that functionally overrides the effect of the bound agonist, a noncompetitive mode; and (*c*) by binding to a separate site induced by bound agonist (such as to a state of the channel promoted by the agonist) i.e., an allosteric mode. Only a competitive mode would predict a perfect correlation, whereas in noncompetitive or allosteric modes, the correlation would depend on the nature of the radioligand used and the degree of the coupling of agonist an antagonist sites. On the other hand, *cis* inhibition did not correlate well with high affinity DHP binding. We therefore propose that the internal effects of DHPs are mediated by an entirely different class of low affinity receptors. Internally accessible DHP receptors may account for the suggestion of Janis et al. (1988) that DHP-like molecules synthesized in the cell may serve as endogenous modulators of Ca channels. Alternatively, they may represent receptors for endogenous peptides that modulate Ca channels and compete

with DHPs (Callewaert et al., 1989). These "internal" sites may serve also as receptors for phenylalkylamines (Hescheler et al., 1982) and may not be present exclusively in the α_1 DHP receptor peptide. Horne et al. (1988) showed in a solubilized and purified preparation of Ca channels of skeletal muscle that ^{45}Ca flux activity assayed in liposomes, and sensitivity to micromolar levels of verapamil, resided in fractions with different peptide compositions. The flux activity required the α_1 peptide, whereas verapamil sensitivity resided in a fraction that included 165 and 55 kDa proteins distinct from α_1 (Horne et al., 1988). This latter fraction was also shown to block the inhibitory action of the G protein G_o. Thus part of its functional domain is likely to be intracellular as shown in the case of the excitatory effects of G_s on t tubule Ca channels (Yatani et al., 1988).

Supported by National Institutes of Health grants GM-36852 and HL-37044, Grants in Aid from the American Heart Association and the Muscular Dystrophy Association of America, and by an Established Investigatorship from American Heart Association to R. Coronado.

Original version received 14 November 1988 and accepted version received 25 April 1989.

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