0026-895X/87/020175-05$02.00/0

MOLECULAR PHARMACOLOGY, 31:175–179

The Relationship Between the Binding Site of [^{3}H]-*d-cis*-Diltiazem and that of Other Non-Dihydropyridine Calcium Entry Blockers in Cardiac Sarcolemma

JOSEPH L. BALWIERCZAK, CARL L. JOHNSON, and ARNOLD SCHWARTZ

Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, Cincinnati, Ohio 45267-0575

Received July 8, 1986; Accepted November 4, 1986

SUMMARY

[^{3}H]-*d-cis*-Diltiazem binds to canine cardiac sarcolemma in a specific, saturable, and reversible manner with a K_D = 58.0 ± 9.5 nM and a receptor site density (maximum binding) of 2.19 ± 0.24 pmol/mg of protein. Bepridil and verapamil, Ca^{2+} channel inhibitors, can completely inhibit this binding at nM concentrations. This inhibition was determined from saturation binding data to be due to a change in affinity of the radioligand, suggesting a competitive interaction between the three drugs. However, in dissociation experiments, both bepridil and verapamil increased the dissociation rate of the radioligand. This effect is uncharacteristic of competitive inhibitors and suggests that bepridil and verapamil regulate [^{3}H]-*d-cis*-diltiazem binding in a negative allosteric fashion through their own distinct binding sites.

The activation of cardiac and smooth muscle contraction occurs with the elevation of intracellular Ca^{2+} mediated by the influx of this cation through a specific membrane channel (1). Intracellular Ca^{2+} is, thus, an important regulator of cardiac and smooth muscle function. Studies on the inhibition of this inward Ca^{2+} current by a new class of drugs called calcium antagonists or calcium entry blockers began with the report of the effects of several of these agents on relaxing coronary smooth muscle (2). Since this initial study, a number of structurally dissimilar classes of calcium antagonists have emerged. Each of the different classes of these drugs has correspondingly different clinical, pharmacological, and electrophysiological effects. Representative members of the main classes of calcium antagonists include the phenylalkylamine derivative, verapamil, the pyrrolidine, bepridil, the benzothiazepine, diltiazem, and the 1,4-dihydropyridine, nifedipine. Since these drugs can influence an important regulator of cardiac and smooth muscle function, namely, Ca^{2+}, their mechanism of action has been the subject of many studies (reviewed in Ref. 3). As a basis for understanding the mechanism by which these drugs inhibit Ca^{2+} channel activity, a number of investigations have been undertaken to determine the relative binding sites for each of the different classes of these drugs. Since all of the different organic calcium antagonists block Ca^{2+} current, it seems reasonable to assume that they all bind to the same site to exert their effect. However, their different chemical structures and pharmacological effects do not support this idea. Direct radioligand binding studies using labeled derivatives of the dihydropyridine class of these drugs have proven that dihydropyridines bind to a site which is separate from the binding sites of the other classes of these drugs (4). However, the nature of the relationship that exists between the binding sites of the different non-dihydropyridine calcium entry blockers has been vigorously debated. Until recently, the primary means of gaining insight into this question was through the use of binding studies with [^{3}H]nitrendipine, a radiolabeled dihydropyridine derivative. The effects of the other non-dihydropyridine calcium antagonists on this binding were found to be complex, thus allowing several interpretations of the results. Murphy *et al.* (5), on the basis of their [^{3}H]nitrendipine binding data from brain tissue, have proposed that all of the non-dihydropyridine calcium antagonists bind to an identical site. Glossmann *et al.* (6), in contrast, have suggested that these calcium antagonists bind to closely linked but separate sites.

The recent introduction of radiolabeled forms of the non-dihydropyridine calcium antagonists has made it possible to determine directly whether there are separate binding sites for each of these classes of calcium antagonists. Using radiolabeled forms of *d-cis*-diltiazem, (±)-bepridil, and the verapamil-like compound (−)-desmethoxyverapamil, Galizzi *et al.* (7) performed saturation binding experiments in rabbit skeletal muscle membranes and concluded that each of these ligands was mutually competitive. In the present work we have used [^{3}H]-*d-cis*-diltiazem to demonstrate specific, saturable, and reversible binding of the ligand to canine cardiac sarcolemma. Our data from saturation binding experiments of [^{3}H]-*d-cis*-diltiazem in the presence of verapamil or bepridil also gave results similar to those of Galizzi *et al.* (7), suggesting a mutual binding site for all three drugs. However, in kinetic experiments, verapamil and bepridil did not act as competitive inhibitors of this

This work was supported in part by National Heart, Lung and Blood Institute Program Project Grant P01-HL-22619-07 (I-C). J. L. B. is a postdoctoral fellow supported by Cardiovascular Training Grant HL-07382-08.

binding, but rather as negative allosteric modulators. Our conclusion is that these three non-dihydropyridine calcium antagonists have closely linked but separate binding sites. Knowledge about the interrelationships of these different binding sites is important, since it will allow appropriate models of the calcium channel to be developed once the primary structure of the channel protein is determined.

Materials and Methods

Isolation and characterization of canine cardiac sarcolemma. Partially purified sarcolemma membranes from canine heart were prepared by a slight modification of the procedure of Van Alstyne *et al.* (8) as described previously (9).

Protein was determined by the method of Lowry *et al.* (10) using bovine serum albumin as standard. The purity of the sarcolemmal preparation was determined by the percentage of total ATPase activity which was ouabain sensitive (Na^+,K^+-ATPase). Sarcolemmal ATPase activity was measured as described by Schwartz *et al.* (11). Routinely, preparations with ATPase activity which were 80–90% ouabain sensitive were used for binding studies.

[^{3}H]-*d-cis*-Diltiazem Binding Assays. For saturation and inhibition binding assays, dog heart sarcolemmal membranes (100 μg of protein) were routinely incubated at 25° in 1 ml of a solution containing 50 mM Tris-maleate buffer (pH 7.8) and varying concentrations of [^{3}H]-*d-cis*-diltiazem (New England Nuclear, Boston, MA). Nonspecific binding was determined by measuring binding in the presence of 10 μM unlabeled *d-cis*-diltiazem. Specific binding was found to be a linear function of the concentration of sarcolemmal membranes between 0.020 and 0.100 mg of membrane protein/ml. Separation of bound ligand from free ligand was accomplished by filtering incubated samples over glass-fiber filters followed by washing of the filters. Initial binding studies showed a high degree of nonspecific binding of [^{3}H]-*d-cis*-diltiazem to glass-fiber filters. Prior incubation of the filters for 2 hr at room temperature in 0.5% polyethylenemine (Sigma Chemical Co., St. Louis, MO) and 2×10^{-5} M unlabeled *d-cis*-diltiazem reduced this nonspecific binding to tolerable levels. However, even after this treatment, the filters displayed specific binding of the radioligand. This was determined by filtering sample blanks (no added protein) containing the same amount of [^{3}H]-*d-cis*-diltiazem with varying amounts of unlabeled *d-cis*-diltiazem. This was the same protocol used in inhibition binding experiments. Samples containing higher concentrations of the unlabeled ligand had lower filter binding than did the samples with lower amounts of the unlabeled drug, indicating displaceable binding to filters. Since this phenomenon could lead to serious error in our binding experiments, we established a procedure to eliminate this binding. By adding 100 μM unlabeled *d-cis*-diltiazem to the washing buffer, we were able to eliminate specific filter binding. After a 2-hr incubation at 25°, samples were diluted to a 5-ml volume with washing buffer (50 mM Tris-HCl, pH 8.0, with 10^{-4} M *d-cis*-diltiazem at 0–2°) and rapidly filtered through Whatman GF/C filters with the aid of a Brandel Cell Harvester (Biomedical Research and Development Laboratories, Gaithersburg, MD). Filters were then washed three times with 5 ml of the washing buffer. The dissociation of [^{3}H]-*d-cis*-diltiazem from the membranes at 0–2° was negligible during the time required for filtration and washing. The filters were placed into scintillation vials; 8 ml of a scintillation cocktail (Research Products International Corp., Elk Grove, IL) were added and radioactivity was counted with a Beckman LS8100 liquid scintillation counter at 40% efficiency. Equilibrium binding experiments were performed in triplicate. K_D and B_{max} values obtained from saturation and inhibition binding data were calculated from total and nonspecific binding data using the LIGAND program of Munson and Rodbard (12).

Kinetic experiments of the dissociation of [^{3}H]-*d-cis*-diltiazem were performed under conditions described for equilibrium binding assays at 10° using a batch method. After equilibrium had been reached, the rate of dissociation of the ligand-receptor complex was monitored at various time points following addition of a large excess of unlabeled *d-cis*-diltiazem (final concentration = 10 μM) alone or along with 100 μM verapamil or 50 μM bepridil. The volume of unlabeled *d-cis*-diltiazem that was added alone or along with bepridil or verapamil was 4% of the total volume. Samples were filtered and washed as above using a Hoefer filtration apparatus (Hoefer Instruments, San Francisco, CA). Radioactivity that was associated with the filters was determined as above.

Materials. [^{3}H]-*d-cis*-Diltiazem (70–80 Ci/mmol) was purchased from New England Nuclear (Boston, Mass). Unlabeled *d-cis*-diltiazem was a gift of Marion Laboratories, Kansas City, MO, courtesy of Dr. R. K. Browne. *l-cis*-Diltiazem was a gift from the Tanabe Seiyaku Co., courtesy of Mr. K. Tanino and Dr. S. Harigaya. Bepridil was supplied by Carter-Wallace Laboratories, courtesy of Dr. Duane Sofia, and verapamil was supplied by Knoll Pharmaceuticals, Whippany, NJ.

Results

Inhibition and equilibrium binding of [^{3}H]-*d-cis*-diltiazem. Analysis of the inhibition of [^{3}H]-*d-cis*-diltiazem binding by unlabeled *d-cis*-diltiazem using the LIGAND program (12) indicated the presence of only a single binding site for the radioligand (Fig. 1). The binding of the radioligand was found also to be influenced by the other classes of calcium antagonists. The complete inhibition of specific [^{3}H]-*d-cis*-diltiazem binding by bepridil and verapamil is seen in Fig. 1. These inhibition binding curves were analyzed using the LIGAND program which yielded K_i values of 32.4 ± 7.9 nM (n = 3), 56.0 ± 11.3 nM (n = 3), and 240 ± 10 nM (n = 3) for verapamil, diltiazem, and bepridil, respectively. The slope of the logit-log plots (not shown) for each of these curves was ~−2.3, indicating that each of these drugs binds to a single class of noninteracting sites. Whether these sites are identical for all three drugs cannot be determined from these data. In addition, nitrendipine, a member of the 1,4-dihydropyridine class of calcium antagonists, influenced [^{3}H]-*d-cis*-diltiazem binding, but to a lesser extent. Fig. 2 shows that binding was stimulated in a dose-dependent manner between 0.1 nM and 5.0 nM nitrendipine. The figure

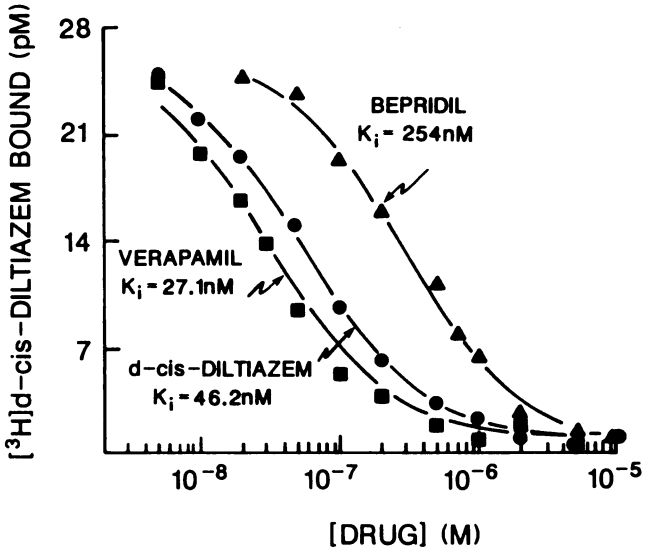

Fig. 1. Competition binding of [^{3}H]-*d-cis*-diltiazem using unlabeled *d-cis*-diltiazem, verapamil, and bepridil as the competing ligands. Inhibition of the binding of 5.0 nM [^{3}H]-*d-cis*-diltiazem by *d-cis*-diltiazem (●), bepridil (▲), and verapamil (■) yielded the respective K_i values 46.2 nM, 254 nM, and 27.1 nM. The *ordinate* represents the amount of total membrane binding, therefore, bepridil and verapamil are shown here to inhibit the same amount of total binding as *d-cis*-diltiazem. This is representative of three experiments each performed in triplicate.

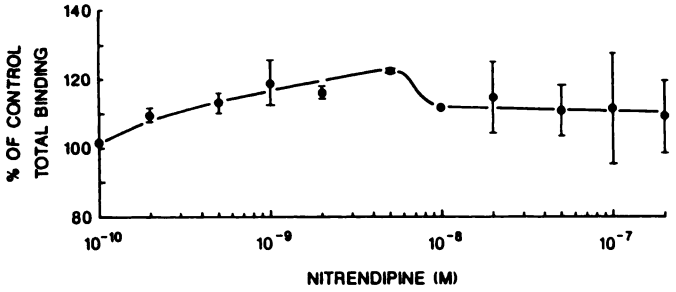

Fig. 2. Competition binding of [^{3}H]-*d-cis*-diltiazem using nitrendipine as the competing ligand. Nitrendipine caused a dose-dependent stimulation of 5.0 nM [^{3}H]-*d-cis*-diltiazem binding between concentrations of 0.1 nM and 5.0 nM. Maximal stimulation was ~20% above control values. See the text for level of significance of effects. Significantly, there was no inhibition of binding over the concentration range tested. This figure represents three experiments each performed in triplicate. *Error bars* represent standard deviation.

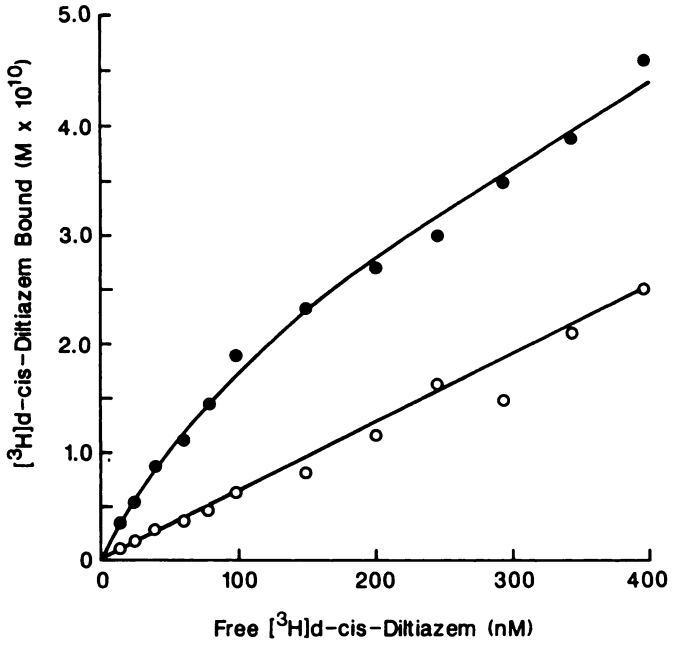

Fig. 3. Saturation binding of [^{3}H]-*d-cis*-diltiazem to cardiac sarcolemma. Total binding of [^{3}H]-*d-cis*-diltiazem was measured over the concentration range of 15 nM to 400 nM (●). Nonspecific binding was measured over the same concentration range in the presence of 10 μM unlabeled *d-cis*-diltiazem (○). The points are the mean of triplicate values and the lines were computer generated from the data using the LIGAND program.

represents three experiments, each performed in triplicate. A one-way analysis of variance with the data obtained using nitrendipine in concentrations from 0.1 nM to 5.0 nM was performed for each of the three experiments. These concentrations were chosen for the analysis since they represented values near the K_D of nitrendipine in this preparation (9). The results of the analysis showed that nitrendipine had a significant effect on binding in each of the three experiments ($p < 0.05$). In two of the experiments, concentrations of nitrendipine from 0.5 nM to 5.0 nM had a significant effect on binding when compared to controls using a Newman-Keuls range test ($p < 0.05$). Significantly, there was no inhibition of binding by nitrendipine up to a concentration of 200 nM.

Fig. 3 illustrates total and nonspecific binding data of [^{3}H]-*d-cis*-diltiazem. Specific binding, which is the difference between total and nonspecific binding, was saturable between [^{3}H]-*d-cis*-diltiazem concentrations of 15 nM and 600 nM. These binding data, analyzed with the LIGAND program (12), indicated a single binding site having an equilibrium dissociation constant (K_D) of 58.9 ± 8.2 nM ($n = 6$) and a maximum binding capacity (B_{max}) of 2.23 ± 0.24 pmol/mg ($n = 6$).

Fig. 4 shows a series of Scatchard plots which illustrate specific binding of [^{3}H]-*d-cis*-diltiazem under control conditions and in the presence of two different concentrations of either bepridil (Fig. 4A) or verapamil (Fig. 4B). These concentrations were approximately the K_i value and 2–3 times the K_i value of bepridil and verapamil, as determined from the inhibition binding experiments in Fig. 1. An increase in the apparent K_D for [^{3}H]-*d-cis*-diltiazem was observed when binding was measured in the presence of 300 nM and 600 nM bepridil (Fig. 4A). When verapamil was present at concentrations of 30 nM and 80 nM, a similar increase was seen (Fig. 4B). These data also show that neither bepridil nor verapamil caused any significant change in the B_{max} of [^{3}H]-*d-cis*-diltiazem. These results are consistent with the idea that bepridil and verapamil are competitive inhibitors of the radioligand. The K_i values obtained for verapamil (32 ± 13 nM) and bepridil (326 ± 58 nM) from the saturation binding data (Fig. 4) were in close agreement with the corresponding values obtained from inhibition binding experiments (Fig. 1).

Dissociation kinetics. The dissociation rate of [^{3}H]-*d-cis*-diltiazem at 25° was determined to be rapid with a $t_{1/2}$ of ~3 min. If any of the conditions of binding were changed such that there was an increase in the dissociation rate of the radioligand, it would have been difficult to accurately measure this change. Standard filtration techniques would not have allowed measuring a rate significantly faster than the control value. In order to determine the dissociation rate under conditions where we might expect an increase in this value, we decided to measure the dissociation rate of [^{3}H]-*d-cis*-diltiazem at 10°. At this temperature the dissociation rate of the radioligand should be slow enough to allow for the accurate measurement of increases in this rate with the use of standard filtration techniques. The dissociation reaction was begun by stopping the forward binding reaction with 10 μM unlabeled diltiazem. The dissociation rate was monophasic over the time period measured (Fig. 5). Total and nonspecific binding were measured at equilibrium and at various times after initiating dissociation. The control dissociation rate constant, k_{-1}, was determined to be 0.016 min^{-1} (Fig. 5). The dissociation rate constant of [^{3}H]-*d-cis*-diltiazem also was determined when dissociation occurred in the presence of 50 μM bepridil or 100 μM verapamil. In these experiments, the ligand-receptor complex was exposed to either verapamil or bepridil at the same time that the dissociation reaction was begun. When bepridil was present during dissociation of the ligand-receptor complex, the k_{-1} value of [^{3}H]-*d-cis*-diltiazem was increased to 0.39 min^{-1}, and in the presence of verapamil, the dissociation rate increased to 0.254 min^{-1} (Fig. 5). This effect is uncharacteristic of competitive antagonism and suggests that the inhibitory effects of verapamil and bepridil on [^{3}H]-*d-cis*-diltiazem binding cannot be explained by simple competition for a mutual binding site.

Discussion

Our data presented here extend previous findings concerning [^{3}H]-*d-cis*-diltiazem binding to cardiac sarcolemma (13). In this report we have demonstrated saturable and reversible binding

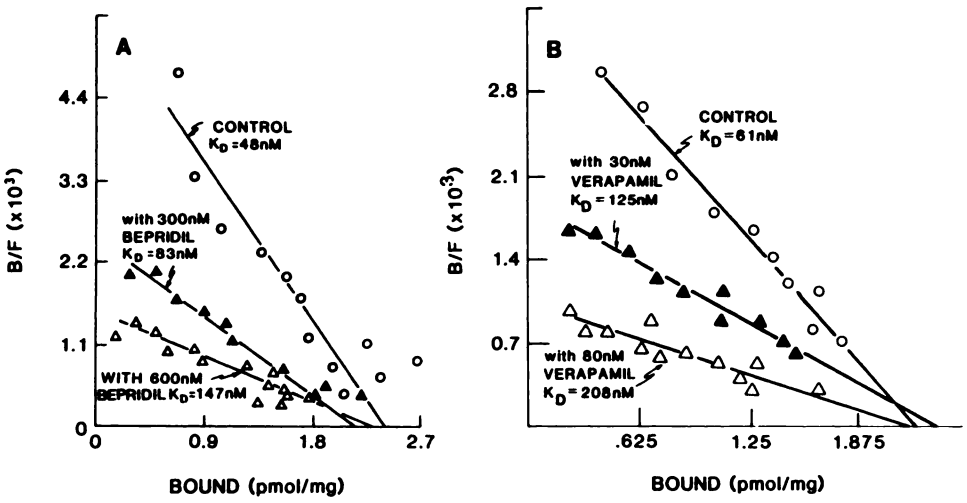

Fig. 4. Saturation binding of [^{3}H]-*d-cis*-diltiazem to cardiac sarcolemma in the absence and presence of bepridil and verapamil. Specific binding data of [^{3}H]-*d-cis*-diltiazem are represented in Scatchard form. A. Saturation binding of [^{3}H]-*d-cis*-diltiazem was carried out as described in the legend to Fig. 3, in the absence (○) and presence of 300 nM bepridil (▲) and 600 nM bepridil (△). The control K_D of 48 nM was increased by 300 nM and 600 nM bepridil to 83 nM and 147 nM, respectively. Nearly identical B_{max} values were obtained under all three conditions. B. Saturation binding of [^{3}H]-*d-cis*-diltiazem carried out in the absence (○) and presence of 30 nM (▲) and 80 nM (△) verapamil. The control K_D of 61 nM was increased by 30 nM and 80 nM verapamil to 125 nM and 208 nM, respectively. Similar B_{max} values were obtained under all three binding conditions. *B/F* represents bound/free with each parameter expressed in molar units. The units for the amount bound on the *x* axis is in pmol/mg of protein.

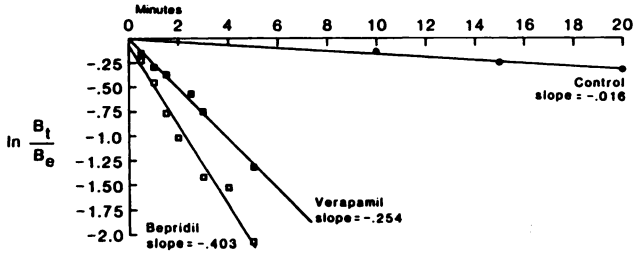

Fig. 5. Dissociation kinetics of [^{3}H]-*d-cis*-diltiazem at 10°. The dissociation of the [^{3}H]-*d-cis*-diltiazem-receptor complex was caused by addition of 10 μM unlabeled diltiazem in the absence (●) and presence of 100 μM verapamil (■) and 50 μM bepridil (□). The rate of dissociation is indicated as the logarithmic transformation of the ratio of specific binding present at the indicated times, B_t, compared to that at equilibrium, B_e. The slopes of the lines are derived from a linear regression analysis of the plotted data and indicate the dissociation rate constants (k_{-1}) under the respective conditions.

of [^{3}H]-*d-cis*-diltiazem to a single site in canine cardiac sarcolemma having an equilibrium K_D of 58.0 ± 9.5 nM (n = 9) and a maximal receptor density (B_{max}) of 2.19 ± 0.24 pmol/mg (n = 9). These values are derived from a combination of saturation and inhibition binding experiments.

Specific binding sites for [^{3}H]-*d-cis*-diltiazem have been demonstrated in rat brain (14), rabbit skeletal muscle transverse tubules (7), and guinea pig skeletal muscle microsomes (15). The K_D value of [^{3}H]-*d-cis*-diltiazem obtained from our studies using cardiac sarcolemma at 25° were identical to those affinities reported for the ligand in brain tissue at 30° (14), and for skeletal muscle at 10° (7). The temperature at which binding is performed is important since the affinity of the ligand is temperature dependent (data not shown). The maximal binding capacity of [^{3}H]-*d-cis*-diltiazem obtained from the data of our saturation experiments was similar to the receptor density obtained for [^{3}H]nitrendipine in this same membrane preparation (9). This suggests that the diltiazem and nitrendipine receptor sites exist in an approximately 1:1 stoichiometry in cardiac sarcolemma.

Our present data clearly suggest that [^{3}H]-*d-cis*-diltiazem is binding to a site closely associated with the calcium channel, since its binding is inhibited by the other non-dihydropyridine calcium antagonists, bepridil and verapamil, and is stimulated by the dihydropyridine calcium antagonist, nitrendipine. It has been known for some time that diltiazem can increase [^{3}H]-1,4-dihydropyridine binding through an unknown positive allosteric mechanism to a variety of tissues (4). Our results are in agreement with these previous findings by showing that this positive allosteric effect is reciprocal. Saturation binding data of [^{3}H]-*d-cis*-diltiazem (Fig. 4) show that verapamil and bepridil cause a change in the apparent K_D of the radioligand, with no change in binding density. This suggests that bepridil and verapamil are competitive inhibitors of the radioligand and that all three drugs bind to the same site. Results similar to these were obtained by Galizzi *et al.* (7) in rabbit skeletal muscle transverse tubules. However, if one examines the effect of these drugs on the dissociation rate of [^{3}H]-*d-cis*-diltiazem, seen in Fig. 5, the decrease in affinity of the radioligand caused by bepridil and verapamil can be explained by their ability to increase its dissociation rate. A competitive inhibitor affects the apparent affinity of another ligand by decreasing its apparent association rate with no effect on its dissociation rate. The results of Fig. 5 suggest that bepridil and verapamil are not competitive inhibitors of [^{3}H]-*d-cis*-diltiazem but, rather, regulate its binding through a negative allosteric effect. The concentrations of bepridil and verapamil used in the dissociation experiments of Fig. 5 were much higher than those needed to fully occupy their respective receptors because we needed a very rapid equilibration of these drugs with their respective binding sites given the rather fast dissociation of the radioligand in these experiments. In addition, we can expect a negative allosteric effect of 10 μM diltiazem (used to initiate the dissociation reaction) on verapamil and bepridil binding, necessitating the use of high concentrations of these drugs to overcome this effect.

Reynolds *et al.* (16) have recently shown that 300 nM diltiazem causes a 40% increase in the k_{-1} of [^{3}H]-(−)-desmethoxyverapamil from a high affinity site in skeletal muscle membranes. These results support our data by indicating that the allosteric relationship between the verapamil- and diltiazem-

binding sites may be reciprocal. These authors propose that a separate lower affinity site labeled by [^{3}H]-(−)-desmethoxyverapamil is the binding site for diltiazem. In addition, these authors show a biphasic dissociation of [^{3}H]-*d-cis*-diltiazem from skeletal muscle, whereas we only see a monophasic dissociation in cardiac tissue. The reason for this difference is not yet known.

There is much evidence gathered to date to support the idea that the dihydropyridine calcium antagonists bind to the calcium channel. Two pieces of evidence which suggest this are that: first, rapid photochemical inactivation of the dihydropyridine channel blockers results in immediate recovery of channel activity at depolarized potentials (17), and second, a dihydropyridine-sensitive calcium channel from purified skeletal muscle transverse tubules has been reconstituted into planar lipid bilayers (18). Since the non-dihydropyridine calcium antagonists can influence [^{3}H]-1,4-dihydropyridine binding to a variety of tissues, it is believed that these drugs also bind to the calcium channel. A recent report by Galizzi *et al.* (7), describing photoaffinity labeling of skeletal muscle transverse tubules with the calcium antagonists [^{3}H]bepridil and [^{3}H]-*d-cis*-diltiazem, showed the specific labeling of a large protein, M_r = 170,000 ± 10,000, by both drugs. These investigators also determined that this same protein was photoaffinity labeled by the dihydropyridine derivative [^{3}H]-(+)-PN200-110. Striessnig *et al.* (19) also showed that the receptor sites for diltiazem, desmethoxyverapamil, and (+)-PN200-110 copurified from transverse tubules of guinea pig skeletal muscle. These reports indicate that the binding sites for both the dihydropyridine and the non-dihydropyridine calcium antagonists probably exist on the same protein molecule.

The structure and physicochemical nature of the calcium antagonist-binding protein/calcium channel, which is being photoaffinity labeled in the types of experiments described above, is the subject of much study (20–22). Structural information will allow the use of modeling to propose the higher orders of the protein's structure as well as the mechanism by which it functions to transport Ca^{2+}. Formation of appropriate molecular models will depend critically on obtaining accurate information about the properties of the calcium channel. Our data, which suggest that the binding sites for the different classes of calcium antagonists are separate and distinct, should help in elucidating the structure of that part of the protein which binds these drugs since we must account for separate binding sites for each of the different classes of calcium antagonists. Moreover, because these binding sites are involved with the functioning of the channel, their structure may give us some clues as to the mechanism by which the protein mediates Ca^{2+} transport.

Recently, two laboratories published results concerning binding of [^{3}H]-*d-cis*-diltiazem to cardiac sarcolemma. An attempt to identify a high affinity binding site for this ligand in rat cardiac sarcolemma by Miwa *et al.* (23) yielded inconclusive results, whereas the report of Garcia *et al.* (24) showed results which were consistent with our conclusion.

Acknowledgments

The authors wish to acknowledge Dr. Pal L. Vaghy for his valuable comments and suggestions during the course of this work and Mr. Edward McKenna and Mr. Richard Wagner for their help with the statistical analyses. We would also like to extend credit to Ms. Gwen Kraft for the art work and to Ms. Liz Wendelmoot for her help in typing the manuscript.

References

1. Reuter, H. Exchange of calcium ions in the mammalian myocardium. *Circ. Res.* **34**:599–605 (1974).
2. Fleckenstein, A., H. Kemmermeier, H. Doring, and H. J. Freund. Zum Wirkungsmechanismus neuartiger koronardilatatoren mit gleichzeitig Sauerstoff-einsparenden Myokard-Effekten. Prenylamin und Iproveratil. Z. Kreislaufforsch. **56**:716–744 (1967).
3. Schwartz, A., and D. J. Triggle. Cellular action of calcium channel blocking drugs. *Annu. Rev. Med.* **35**:325–339 (1984).
4. Triggle, D. J., and R. A. Janis. Calcium channel antagonists: new perspectives from the radioligand binding assay. *Modern Methods Pharmacol.* **2**:1–28 (1984).
5. Murphy, K. M. M., R. J. Gould, B. L. Largent, and S. H. Snyder. A unitary mechanism of calcium antagonist drug action. *Proc. Natl. Acad. Sci. USA* **80**:860–864 (1983).
6. Glossmann, H., D. R. Ferry, A. Goll, and M. Rombusch. Molecular pharmacology of the calcium channel: evidence for subtypes, multiple drug-receptor sites, channel subunits, and the development of a radioiodinated 1,4-dihydropyridine calcium channel label, [^{125}I]iodipine. *J. Cardiovasc. Pharmacol.* **6**:S608–S621 (1984).
7. Galizzi, J.-P., M. Borsotto, J. Barhanin, M. Fosset, and M. Lazdunski. Characterization and photoaffinity labeling of receptor sites for the Ca^{2+} channel inhibitors *d-cis*-diltiazem, (±)-bepridil, desmethoxyverapamil, and (+)PN200-110 in skeletal muscle transverse tubule membranes. *J. Biol. Chem.* **261**:1393–1397 (1986).
8. Van Alstyne, E., R. M. Burch, R. G. Knickelbein, R. T. Hungerford, E. J. Gower, J. G. Webb, S. L. Poe, and G. E. Lindenmayer. Isolation of sealed vesicles highly enriched with sarcolemma markers from canine ventricle. *Biochim. Biophys. Acta* **602**:131–143 (1980).
9. Balwierczak, J. L., I. L. Grupp, G. Grupp, and A. Schwartz. Effects of bepridil and diltiazem on [^{3}H]nitrendipine binding to canine cardiac sarcolemma. Potentiation of pharmacological effects of nitrendipine by bepridil. *J. Pharmacol. Exp. Ther.* **237**:40–48 (1986).
10. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
11. Schwartz, A., K. Nagano, M. Nakao, G. E. Lindenmayer, J. C. Allen, and H. Matsui. The sodium and potassium activated adenosinetriphosphatase system. *Methods Pharmacol.* **1**:361–388 (1971).
12. Munson, P. J., and D. Rodbard. A versatile computerized approach for characterization of ligand-binding systems. *Anal. Biochem.* **107**:220–239 (1980).
13. Balwierczak, J. L., and A. Schwartz. Specific binding of [^{3}H]*d-cis*-diltiazem to cardiac sarcolemma and its inhibition by calcium. *Eur. J. Pharmacol.* **116**:193–194 (1985).
14. Schoemaker, H., and S. Z. Langer. [^{3}H]Diltiazem binding to calcium channel antagonists recognition sites in rat cerebral cortex. *Eur. J. Pharmacol.* **111**:273–277 (1985).
15. Glossmann, H., T. Linn, M. Rombusch, and D. R. Ferry. Temperature-dependent regulation of *d*-cis-[^{3}H]diltiazem binding to Ca^{2+} channels by 1,4-dihydropyridine channel agonists and antagonists. *FEBS Lett.* **160**:226–232 (1983).
16. Reynolds, I. J., A. M. Snowman, and S. H. Snyder. (−)-[^{3}H]Desmethoxyverapamil labels multiple calcium channel modulator receptors in brain and skeletal muscle membranes: differentiation by temperature and dihydropyridines. *J. Pharmacol. Exp. Ther.* **237**:731–738 (1986).
17. Gurney, A. M., J. M. Nerbonne, and H. A. Lester. Photoremoval of nifedipine reveals the likely mechanism of action of this Ca^{2+} antagonist. *Biophys. J.* **45**:35a (1984).
18. Affolter, H., and R. Coronado. Agonists Bay-K 8644 and CGP-28392 open calcium channels reconstituted from skeletal muscle transverse tubules. *Biophys. J.* **48**:341–347 (1985).
19. Striessnig, J., A. Goll, K. Moosburger, and H. Glossmann. Purified calcium channels have three allosterically coupled drug receptors. *FEBS Lett.* **197**:204–210 (1986).
20. Borsotto, M., J. Barhanin, M. Fosset, and M. Lazdunski. The 1,4-dihydropyridine receptor associated with the skeletal muscle voltage-dependent Ca^{2+} channel. *J. Biol. Chem.* **260**:14255–14263 (1985).
21. Curtis, B., and W. A. Catterall. Purification of the calcium antagonist receptor of the voltage-sensitive calcium channel from skeletal muscle transverse tubules. *Biochemistry* **23**:2113–2118 (1984).
22. Ferry, D. R., K. Kampf, A. Goll, and H. Glossmann. Subunit composition of skeletal muscle transverse tubule calcium channels evaluated with the 1,4-dihydropyridine photoaffinity probe, [^{3}H]azidopine. *EMBO J.* **4**:1933–1940 (1985).
23. Miwa, N., H. Kanaide, J. Nishimura, and M. Nakamura. Binding of [^{3}H]-nitrendipine and [^{3}H]-diltiazem to rat myocardial sarcolemma. *Arzneim.-Forsch.* **36**:1059–1062 (1986).
24. Garcia, M. L., V. F. King, P. K. S. Siegl, J. P. Reuben, and G. J. Kaczorowski. Binding of Ca^{2+} entry blockers to cardiac sarcolemmal membrane vesicles. *J. Biol. Chem.* **261**:8146–8157 (1986).

Send reprint requests to: Dr. Arnold Schwartz, Department of Pharmacology and Cell Biophysics, University of Cincinnati College of Medicine, 231 Bethesda Avenue, Cincinnati, OH 45267-0575.