Analysis of the Dihydropyridine Receptor Site of L-type Calcium Channels by Alanine-scanning Mutagenesis*

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The dihydropyridine Ca\(^{2+}\) antagonist drugs used in the therapy of cardiovascular disorders inhibit L-type Ca\(^{2+}\) channels by binding to a single high affinity site. Photoaffinity labeling and analysis of mutant Ca\(^{2+}\) channels implicated the IIIS6 and IVS6 segments in high affinity binding. The amino acid residues that are required for high affinity binding of dihydropyridine Ca\(^{2+}\) channel antagonists were probed by alanine-scanning mutagenesis of the \(\alpha_{L_{C}}\) subunit, transient expression in mammalian cells, and analysis by measurements of ligand binding and block of Ba\(^{2+}\) currents through expressed Ca\(^{2+}\) channels. Eleven amino acid residues in transmembrane segments IIIS6 and IVS6 were identified whose mutation reduced the affinity for the Ca\(^{2+}\) antagonist PN200-110 by 2–25-fold. Both amino acid residues conserved among Ca\(^{2+}\) channels and those specific to L-type Ca\(^{2+}\) channels were found to be required for high affinity dihydropyridine binding. In addition, mutation F1462A increased the affinity for the dihydropyridine Ca\(^{2+}\) antagonist PN200-110 by 416-fold with no effect on the affinity for the Ca\(^{2+}\) agonist Bay K8644. The residues in transmembrane segments IIIS6 and IVS6 that are required for high affinity binding are primarily aligned on single faces of these two \(\alpha\) helices, supporting a "domain interface model" of dihydropyridine binding and action in which the IIIS6 and IVS6 interact to form a high affinity dihydropyridine receptor site on L-type Ca\(^{2+}\) channels.

Voltage-gated Ca\(^{2+}\) channels mediate Ca\(^{2+}\) influx in response to membrane depolarization and thereby initiate cellular activities such as secretion, contraction, and gene expression. Several types of voltage-gated Ca\(^{2+}\) channels have been distinguished by their physiological and pharmacological properties and have been designated L, N, P/Q, R, and T (for review see Refs. 1–3). L-type Ca\(^{2+}\) channels are the molecular targets for the dihydropyridine, phenylalkylamine, and benz(othia)zepine classes of calcium channel blockers that are widely used in the therapy of cardiovascular diseases. These drugs are thought to bind to three separate but allosterically coupled receptor sites on L-type Ca\(^{2+}\) channels (4, 5).

The L-type Ca\(^{2+}\) channels consist of the pore-forming \(\alpha_{L}\) subunits of 190–250 kDa in association with disulfide-linked \(\alpha_{L}\delta\) subunits of approximately 140 kDa, intracellular \(\beta\) subunits of 55–72 kDa, and, for the skeletal muscle L-type channel, an additional transmembrane \(\gamma\) subunit of 33 kDa (for review see Ref. 6). The \(\alpha_{L}\) subunits confer the characteristic pharmacology and functional properties of each channel type, but their function is modulated by association with the auxiliary subunits. The pore-forming \(\alpha_{L}\) subunits can be divided into two distinct families, L-type and non-L-type, which share less than 40% amino acid identity. The L-type Ca\(^{2+}\) channel \(\alpha_{L}\) subunit family includes skeletal muscle (\(\alpha_{L_{S}}\)) (7), cardiac/smooth muscle/neuronal (\(\alpha_{L_{C}}\)) (8–10), and endocrine/neuronal (\(\alpha_{L_{D}}\)) (11, 12) isoforms. The non-L-type \(\alpha_{L}\) subunit family also consists of at least three distinct gene products expressed primarily in neurons: N-type (\(\alpha_{N_{H}}\)) (13), P/Q-type (\(\alpha_{P/Q_{A}}\)) (14, 15), and R-type (\(\alpha_{R_{B}}\)) (16).

The dihydropyridines (DHPs) are allosteric modulators that act on L-type Ca\(^{2+}\) channels as either agonists or antagonists (reviewed in Refs. 17 and 18). Charged DHPs are thought to traverse an extracellular pathway to gain access to the DHP receptor site located within the lipid bilayer 11–14 Å from the extracellular surface of the cell membrane (19–22). Photoactive DHPs specifically label the \(\alpha_{L}\) subunit of the Ca\(^{2+}\) channel (23–29). The predominant site of labeling corresponds to transmembrane segment IIIS6 and a portion of the extracellular segment connecting IIIS5 and IIIIS6, and approximately 15–30% of the total photolabeling is incorporated into a peptide fragment composed primarily of transmembrane segment IVS6 (30–33). In addition to these membrane-associated regions, a site in the intracellular carboxyl-terminal domain has been photoaffinity-labeled by photoactive DHPs (34). Analysis of chimeric Ca\(^{2+}\) channels implicated the IIIIS5 (35), IIIIS6 (35), and IVS6 (35–37) transmembrane segments in DHP binding, in general agreement with previous photoaffinity labeling results (30, 31).

Site-directed mutagenesis of single amino acid residues that differ between L-type and non-L-type Ca\(^{2+}\) channels revealed multiple amino acid residues on one side of the IIIIS6 and IVS6 putative transmembrane \(\alpha\) helices that are important determinants of high affinity binding of DHP agonists and antagonists to L-type Ca\(^{2+}\) channels (37, 38). In addition, mutation of a Tyr residue (Tyr\(^{1048}\) in \(\alpha_{L_{S}}\)) in segment IIIIS6 that is conserved in all Ca\(^{2+}\) subtypes was found to have the largest effect on DHP affinity (38). Mutation of this residue to Phe caused a 12-fold reduction in binding affinity, and mutation to Ala prevented measurable high affinity DHP binding. In the experiments reported here, we have used alanine-scanning mutagenesis to map both conserved and L-type-specific amino acid residues that contribute to the high affinity of the DHP receptor site.

EXPERIMENTAL PROCEDURES

Materials—taSA-201 cells were provided by Dr. Robert DuBridge (Cell Genesis, Foster City, CA). cDNA encoding the \(\alpha_{L}\) subunit cloned from

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1 The abbreviation used is: DHP, dihydropyridine.
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rabbit skeletal muscle calcium channel was provided by Drs. Steven B. Ellis, Michael M. Harpold (Salk Institute Biotechnology/Industrial Associates, Inc., La Jolla, CA), and Arnold Schwartz (University of Cincinnati College of Medicine, Cincinnati, OH). Zem 228C was provided by Dr. Eileen Mulvihill (Zymogenetics, Seattle, WA). Full-length αCII in pGem229 was provided by Dr. Terry P. Snutch (University of British Columbia, Vancouver, Canada). It encodes an isoform of αC2 cloned from rat brain (10). The expression plasmid pReCMV was obtained from Invitrogen Corporation (San Diego, CA). Phage M13mp19 was obtained from Life Technologies, Inc. (+)-[3H]PN200-110 (76–86 Ci/mmol) was purchased from NEN Life Science Products. (+)-PN200-110 was obtained from Research Biochemicals International (Natick, MA).

Construction of Mutant Ca2+ Channels—For the construction of the mutant αC2 subunits (10), a 1.5-kilobase Esf fragment of the αC2 subunit of the rat brain Ca2+ channel was subcloned into the bacteriophage M13mp19 for recovery of single-stranded DNA template. The resulting construct was used as a template for the generation of single-stranded DNA for introduction of mutations into transmembrane segment IVS6 of αC2. These mutations were transferred to the full-length αC2 subunit cDNA by subcloning the 272-base pair DroIII/DroIII fragment. Mutations in transmembrane segment III/IV were made with a single-stranded DNA template containing the 1.7-kilobase AvrII/EcoRI fragment of the αC1C cDNA. The III/IV mutations were transferred into the full-length αC1C construct using the 1.5-kilobase SpcIDroIII and the 272-base pair DroIII/DroIII fragments in a three-way ligation. Reverse-directed mutagenesis was performed using established procedures (39), mutations were verified by sequence analysis of approximately 200 base pairs surrounding the site of mutagenesis, and the integrity of the full-length mutant Ca2+ channel cDNA was confirmed by extensive restriction digest analysis.

Expression of Ca2+ Channels—Human tsa-201 cells, a simian virus 40 (SV40) T-antigen expressing derivative of the human embryonic kidney cell line HEK293, were maintained in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Life Technologies, Inc.) enriched with 10% fetal bovine serum. Human tsa-201 cells were cotransfected with wild type or mutant αC1C and cDNA encoding the β1 (40) and αδ Ca2+ channel subunits such that the molar ratio of the plasmids was 1:1:1. Cells were transfected by calcium phosphate precipitation according to the procedures outlined by Margolade et al. (41), and cells were harvested 30–40 h following transfection.

Preparation of Membranes—Cells were washed two times, scraped, and homogenized using a glass-teflon homogenizer in Buffer A (50 mM Tris, 100 μM phenylmethanesulfonfluoride, 100 μM benzamidine, 1.0 μg pepstatin A, 1.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin, pH 8.0). The homogenate was centrifuged at 700 g for 10 min, the supernatant was discarded, and the supernatant was centrifuged 30 min at 100,000 × g. The supernatant was discarded, and the membrane pellet was washed and homogenized in Buffer A. The resulting membrane homogenate was divided into aliquots and stored at −80 °C for up to 3 months with no detectable loss of (±)-[3H]PN200-110 binding activity.

Radioligand Binding—Equilibrium binding assays were performed in triplicate in 150 mM Tris, 100 μM phenylmethanesulfonfluoride, 100 μM benzamidine, 1.0 μg pepstatin A, 1.0 μg/ml leupeptin, and 2.0 μg/ml aprotinin, pH 8.0 with 20–200 μg of membrane protein, 0.01–10 nM (±)-[3H]PN200-110 and 1 mM Ca2+ at 32 °C for 180–210 min. Nonspecific binding was determined in the presence of 1 μM (±)-PN200-110, and bound and free ligand were separated by vacuum filtration over GF/C glass fiber filters. Filters were washed using ice-cold wash buffer (10 mM Tris, 1% polyethylene glycol 8000, 0.1% bovine serum albumin, 0.01% Triton X-100, pH 8.0), and bound radioactivity was detected by liquid scintillation counting. Dissociation constants (Kd) were determined using the radioligand data analysis program LIGAND. All data are means ± S.E.

Electrophysiology— Cultures of tsa-201 cells at 75% confluence in 35-mm cell culture dishes were transfected with a total of 4 μg of DNA consisting of an equimolar ratio of the three channel subunit cDNAs and 0.8 μg of CDS cDNA. After the addition of CaPO4-DNA, cells were incubated overnight at 37 °C in 5% CO2. Twenty h after transfection, the cells were removed from culture dishes using 2 mM EDTA in phosphate-buffered saline and replated at low density for electrophysiological analysis. Transfectants were identified by labeling with anti-CDS antibody-coupled Dynabeads (Dynal, Inc.) or by Barium currents through L-type Ca2+ channels were recorded using the whole cell configuration of the patch clamp technique. Patch electrodes were pulled from VWR micropipettes and fire-polished to produce an inner tip diameter of 4–6 μm. Currents were recorded using a List EPC-7 patch clamp amplifier and filtered at 2 kHz (pole Bessel filter, −3 db). Voltage pulses were applied, and data were acquired using Fastlab software (Indec Systems). Linear leak and capacitance currents have been subtracted using an on-line P/4 subtraction paradigm. (+)-PN200-110 was applied to cells by the addition of 0.2 ml of a 6× stock to 1 ml bath. Inhibition of Ca2+ channel current (carried by Ba2+) during application of (+)-PN200-110 was monitored by 100-ms depolarizations to +10 mV from a holding potential of −80 mV applied every 10 s until block had reached steady-state (1–3 min). The bath saline contained 150 mM Tris, 2 mM MgCl2, and 10 mM BaCl2. The intracellular saline contained 130 mM N-methyl-d-glucamine, 10 mM EGTA, 60 mM HEPES, 2 mM MgATP, and 1 mM MgCl2. The pH of both solutions was adjusted to 7.3 with methanesulfonic acid. All experiments were performed at room temperature (20–23 °C).

RESULTS

Transmembrane segments III/IV and IVS6 contain important molecular determinants for high affinity binding of DHPs as determined by photoaffinity labeling (Fig. 1A; Refs. 30 and 31) and by analysis of chimeric and mutant Ca2+ channels (Fig. 1B; Refs. 35–38). Previous molecular biological studies that support this conclusion have focused almost exclusively on amino acid residues that are present in DHP-sensitive L-type Ca2+ channels but not in DHP-insensitive non-L-type channels.

In the present experiments, alanine-scanning mutagenesis of single amino acid residues in transmembrane segments III/IV and IVS6 of αC1C was employed to identify all of the amino acids, both conserved and t-type-specific, that are important determinants for DHP binding. Most of the amino acids in III/IV and IVS6 of αC1C were systematically changed to Ala and coexpressed with αδ and β1b in tsa-201 cells (Fig. 1, C and D, shaded boxes). To assess the relative importance of Ala1575 and Ala1647, these amino acids were changed to Pro and Ser, respectively, as found in DHP-insensitive Ca2+ channels. Ala was chosen for these experiments because its substitution in α helices in the core of proteins has minimal effects on secondary structure (42). Substitution of Ala is therefore expected to reduce the hydrophobicity and size of the amino acid residue in each position in these putative α helices without causing global conformational changes.

Wild type and mutant Ca2+ channels were expressed transiently in tsa-201 cells, membrane preparations were isolated by differential centrifugation, DHP binding was measured in filter binding assays using the radiolabeled DHP antagonist (+)-[3H]PN200-110, and Kd values for high affinity DHP binding were determined by Scatchard analysis. A high ratio of specific to nonspecific binding was observed in binding experiments with both wild type Ca2+ channels (not shown) and mutant channels having substantially reduced affinity (for example, mutant N1472A; Fig. 2A). The linearity of the Scatchard plots (Fig. 2B) indicates a single class of high affinity binding sites with a Kd of 55 psi for wild type and 470 psi for the mutant N1472A.

Mutation of several amino acid residues in transmembrane segments III/IV and IVS6 to Ala had large effects on DHP binding affinity (Fig. 3). Of the mutations in III/IV, the largest effects on PN200-110 binding were observed when the l-type-specific residues Ile1553, Ile1556, Met1609, and Met1611 were changed to Ala, resulting in Kd values that are larger than wild type by 6.2-, 17-, 3.5-, and 9.6-fold, respectively (Fig. 3A). In addition, mutation of three residues in IVS6 that are conserved between l-type and non-L-type Ca2+ channels to Ala had large effects on DHP binding. The mutants F1158A and P1159A have Kd values that are 1.5- and 4.3-fold larger than wild type, respectively. The mutant Y1152A, which corresponds to the conserved residue Tyr1048 in α1C, which we previously showed was essential for DHP binding (38), exhibits no detectable DHP binding at concentrations of (+)-[3H]PN200-110 up to 25 nM.

Of the amino acid residues screened by alanine-scanning mutagenesis in transmembrane segment IVS6, mutations Y1463A, M1464A, and I1471A, which affect t-type-specific res-
Fig. 1. Location of the DHP receptor site. A, a transmembrane folding model for domains III and IV of the l-type Ca\(^{2+}\) channel \(a_1\) subunit is used to illustrate the peptide segments critical for DHP binding. Bold and shaded regions refer to segments of the receptor site found to be important for the actions of DHPs by photoaffinity labeling (30–32). Numbers refer to the amino acid residues in \(a_{1C}\), analogous to the NH\(_2\) and COOH termini of the photoaffinity-labeled peptides. B, a transmembrane folding model for domains III and IV of the l-type Ca\(^{2+}\) channel \(a_1\) subunit is used to illustrate the peptide segments critical for DHP binding. Bold and shaded regions refer to segments of the receptor site that were identified using chimeric Ca\(^{2+}\) channels (35–37). Numbers refer to amino acid positions in the sequence of \(a_{1C}\) that are analogous to those identified by site-directed mutagenesis and radioligand binding experiments as being critical for the binding of DHP agonists and antagonists (38). C and D, sequence alignment of transmembrane segments I\(\alpha IS\) (C) and IV\(\alpha IS\) (D) from DHP-sensitive (\(a_{15}\), \(a_{1C}\), and \(a_{1D}\)) and DHP-insensitive (\(a_{1A}\), \(a_{1B}\), and \(a_{1C}\)) channel \(a_1\) subunits. Asterisks refer to t-type-specific amino acids in I\(\alpha IS\) and IV\(\alpha IS\) of \(a_{1C}\) that were analyzed by mutation, expression, and radioligand binding previously (38). Also indicated by an asterisk is the conserved Tyr1462, which was the most essential residue identified by Peterson et al. (38). Shaded boxes refer to amino acids that were analyzed by alanine scanning mutagenesis and radioligand binding in this study. Circles refer to amino acids found to be critical for the binding and/or actions of DHP agonists and antagonists. F1462A is identified by a white circle.

Wound and N1472A, which affects a conserved residue, exhibited the largest effects, with \(K_d\) values that were larger than wild type by 2.9-, 1.6-, 2.7-, and 9-fold, respectively (Fig. 3B). The comparatively small effects of mutation of residues in IV\(\alpha IS\) compared with I\(\alpha IS\) suggest that this transmembrane segment is on the periphery of the DHP receptor site and has relatively weak interactions with bound DHPs compared with I\(\alpha IS\).

We were unable to detect specific DHP binding to the \(a_{1C}\) mutants Y1152A, Y1458A, and F1462A using concentrations of (+)-\[^{3}H\]PN200-110 up to 25 nM. To determine if these mutants can form functional Ca\(^{2+}\) channels and to measure their affinity for DHPs, these mutants were expressed and analyzed electrophysiologically (Fig. 4). The mutant Y1458A did not express Ba\(^{2+}\) currents, so it is likely that the lack of DHP binding observed with this mutant is caused by failure to express functional Ca\(^{2+}\) channels. The mutants Y1152A and F1462A both expressed Ba\(^{2+}\) currents but less effectively than wild type (Fig. 4, B and C). The voltage dependence of activation of these mutants was comparable with that of wild type as assessed from current-voltage relationships. In contrast, the voltage dependence of inactivation was shifted for these mutant Ca\(^{2+}\) channels (Fig. 4D). For mutant Y1152A, the voltage dependence of inactivation was shifted to more negative membrane potentials and became significantly less steep than wild type. For mutant F1462A, the voltage dependence of inactivation was shifted 10 mV to more positive membrane potentials. As DHP antagonists bind with higher affinity to inactivated Ca\(^{2+}\) channels, changes in the extent of inactivation at the membrane potential of our experiments could influence DHP binding and block. However, as shown below, the changes in DHP affinity caused by these two mutations are in the opposite directions from those expected on the basis of the changes in the voltage dependence of inactivation so the changes in affinity are likely to result from molecular changes in the DHP binding site rather than from indirect allosteric effects due to the change in inactivation gating.

The DHP antagonist PN200-110 inhibits wild type and mutant Ca\(^{2+}\) channels in a concentration-dependent manner (Fig. 4, A–C). Analysis of concentration-effect relationships indicates that the IC\(_{50}\) for inhibition of Y1152A by PN200-110 at −60 mV is approximately 25-fold higher than wild type (Fig. 4E). Thus, Tyr\(^{151}\) is indeed an important determinant for DHP binding. This large decrease in binding affinity coupled with low levels of expression is likely to prevent detection of specific (+)-\[^{3}H\]PN200-110 binding to this mutant.

In contrast, the IC\(_{50}\) for block of mutant F1462A by PN200-110 was decreased from 6.8 ± 1.2 nM to 16 ± 5.8 pM, indicating that this mutation results in an \(a_{1C}\) subunit with an affinity for PN200-110 at −60 mV that is at least 400-fold higher than wild type (Fig. 4E). The Ba\(^{2+}\) currents for this mutant are very small and fewer cells express detectable Ba\(^{2+}\) current. Therefore, the failure to detect (+)-\[^{3}H\]PN200-110 binding to F1462A is likely due to a combination of low expression levels and expression in fewer cells rather than a reduction in DHP affinity. Surprisingly, the effect of the DHP agonist Bay K8644 to enhance Ca\(^{2+}\) currents was not altered in this mutant (EC\(_{50}\) = 151 ± 84 pM for wild type; EC\(_{50}\) = 111 ± 45 pM for F1462A, n = 3). Thus, mutation at this position has a large and specific effect on high affinity binding of a DHP antagonist without
effect on agonist binding and action. Analysis of additional mutations at this position will be of interest to further define the structural basis for the specific increase in affinity for DHP antagonists.

**DISCUSSION**

Mapping of the DHP Receptor Site by Alanine-scanning Mutagenesis—A variety of techniques have been used to localize the DHP receptor site. Results from experiments using charged DHPs, photoreactive DHPs, chimeric Ca\(^{2+}\) channels, and mutations of L-type-specific amino acid residues all indicate that the core of the DHP receptor site is formed primarily by amino acid residues in IIIS6 and IVS6 (Fig. 1, A and B). In this study, most of the amino acids in IIIS6 and IVS6 of a\(_{1C}\) were systematically changed to Ala, and intrinsic Ala residues were changed to the amino acids present in corresponding positions in non-L-type Ca\(^{2+}\) channels (see Fig. 1, C and D, shaded boxes). The amino acid residues found by alanine-scanning mutagenesis to be important for DHP binding are indicated with circles in the second lines of panels C and D of Fig. 1, and they are compared with those identified by mutations of L-type-specific residues in our previous studies in the top lines of panels C and D in Fig. 1 (38). Our results using alanine-scanning mutagenesis have identified four new conserved amino acid residues and two new L-type-specific amino acid residues within transmembrane segments IIIS6 and IVS6 whose mutation has significant effects on DHP binding affinity. The positions of these critical amino acid residues within the IIIS6 and IVS6 \(\alpha\) helices are illustrated in Fig. 5 where black residues indicate a reduction of DHP binding affinity by 5-fold or more and shaded residues indicate a significant reduction in binding affinity that is less than 5-fold by mutation to Ala.

In segment IIIS6, the largest effects were observed when amino acid residues Tyr\(^{1152}\), Ile\(^{1153}\), Ile\(^{1156}\), and Met\(^{1161}\) were changed to Ala, resulting in \(K_d\) values that are larger than wild type by 25-, 6.2-, 17-, and 9.6-fold, respectively (Fig. 5, black residues). The mutants Phe\(^{1158}\), Phe\(^{1159}\), and Met\(^{1160}\) had \(K_d\) values for \((+)^{-}[^{3}H]PN200-110\) binding that were 3.5–4.5-fold higher than wild type (Fig. 5, shaded residues). The COOH-terminal portion of IIIS6 including residues Phe\(^{1158}\), Phe\(^{1159}\), Met\(^{1160}\), and Met\(^{1161}\) was not analyzed in our previous studies; therefore, all of these amino acids are newly identified residues that are important determinants of high affinity DHP binding in transmembrane segment IIIS6.

In segment IVS6, mutations of residues Tyr\(^{1463}\), Ile\(^{1471}\), and Asn\(^{1472}\) had the largest effects, with increases in \(K_d\) values for \((+)^{-}[^{3}H]PN200-110\) of 2.9–1.6–2.7–9-fold, respectively. In addition, mutation F1462A caused a large increase in affinity for a DHP antagonist with no change in affinity for the DHP agonist Bay K8644. In our previous study, all mutations of L-type-specific amino acid residues had similar effects on the binding affinity for DHP agonists and antagonists (38). Thus, Phe\(^{1462}\) may have unique interactions with DHP antagonists that both determine their affinity and differentiate between agonists and antagonist drugs.
Asn\textsuperscript{1472} in transmembrane segment IVS6 is conserved between Ca\textsuperscript{2+} and Na\textsuperscript{+} channels. When this residue is changed to Ala in Na\textsuperscript{+} channels (N1769A), the resulting mutant has a 15-fold increased affinity for local anesthetic binding to the resting state of the channel (43). It was hypothesized that this mutation creates a local conformation of the drug receptor site in the resting state of the channel which resembles the high affinity local conformation of that receptor site in the inactivated state (43). Because the affinity for drug binding is highest in the inactivated states for both Na\textsuperscript{+} and Ca\textsuperscript{2+} channels, the increase in affinity for block of resting calcium channels by DHPs observed with the F1462A mutation in this work may have a similar molecular basis as the state-dependent increase in affinity for block of resting calcium channels by local anesthetics. These results suggest that DHP binding involves conserved amino acid residues that are important for common aspects of the structure and function of all voltage-gated Ca\textsuperscript{2+} channels. These conserved residues are likely to be largely responsible for the low affinity binding of DHPs found in non-L-type Ca\textsuperscript{2+} channels (44).

We have also identified several L-type-specific amino acid residues in IIIIS6 and IVS6 that are critical molecular determinants for DHP binding. Alteration of four L-type-specific residues in IIIIS6 (Ile\textsuperscript{1153}, Ile\textsuperscript{1156}, Met\textsuperscript{1160}, and Met\textsuperscript{1161}) and three L-type-specific residues in IVS6 (Tyr\textsuperscript{1463}, Met\textsuperscript{1464}, and Ile\textsuperscript{1471}) resulted in channels with significantly increased \( K_d \) values for (+)-[\textsuperscript{3}H]PN200-110 binding. It is likely that these residues are largely responsible for the high affinity of L-type Ca\textsuperscript{2+} channels for DHPs and for the requirement of the IIIIS6 and IVS6 transmembrane segments for the transfer of high affinity DHP binding to non-L-type Ca\textsuperscript{2+} channels as demonstrated in experiments with channel chimeras (35–38). Recent studies also implicate two additional L-type-specific amino acid residues in transmembrane segment IIISS5 in high affinity DHP binding and in the transfer of high affinity DHP binding to non-L-type Ca\textsuperscript{2+} channels (45).

The DHP Receptor Site Involves Both Conserved and L-type-specific Amino Acid Residues—The largest effects on DHP binding affinity were caused by alteration of conserved amino acid residues. The largest decrease in affinity (25-fold) was seen for mutation of Tyr\textsuperscript{1552} in IIIIS6. Mutation of the conserved residue Asn\textsuperscript{1472} in IVS6 also caused a major (9-fold) decrease in DHP binding affinity. Alteration of a third conserved residue, Phe\textsuperscript{1462} resulted in a 416-fold decrease in IC\textsubscript{50} for block by PN200-110, consistent with greatly increased DHP affinity of resting Ca\textsuperscript{2+} channels. These results suggest that DHP binding involves conserved amino acid residues that are important for common aspects of the structure and function of all voltage-gated Ca\textsuperscript{2+} channels. These conserved residues are likely to be largely responsible for the low affinity binding of DHPs found in non-L-type Ca\textsuperscript{2+} channels (44).

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The Domain Interface Model of Ca\textsuperscript{2+} Agonist and Antagonist Binding—All three classes of Ca\textsuperscript{2+} antagonist drugs and the DHP Ca\textsuperscript{2+} agonist drugs are allosteric ligands that exhibit state-dependent binding and are influenced in a reciprocal manner by binding of the other classes of drugs to distinct but interacting sites. Their effects on Ca\textsuperscript{2+} channel function and their interactions with each other are similar to the actions of allosteric effectors on enzymes. In most well studied cases, binding of allosteric effectors to enzymes is thought to take place at subunit interfaces (46–48). Similarly, strong evidence now indicates that the agonists of pentameric nicotinic acetylcholine receptors bind at sites that are formed at the interfaces of the \( \alpha \) subunits with the \( \beta \) and \( \delta \) subunits (49). Thus, there is increasingly strong precedent for binding of allosteric ligands at structural interfaces, possibly because the structure of multimeric proteins is most flexible in those regions. Our results
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alter protein-protein interactions at the interface between domains III and IV. Thus, the modulation of Ca\(^{2+}\) channel function by all three classes Ca\(^{2+}\) antagonist drugs appears to be structurally analogous to the regulation of allosteric enzymes by their effectors.

Allosteric Interactions among Dihydropyridines, Phenylalkylamines, and Benz(othi)azepines—Ligand binding studies indicate that the phenylalkylamines, benz(othi)azepines, and DHPs bind to three separate receptor sites that interact allosterically (4, 5). This requires that all three classes of drugs be able to occupy their receptor sites simultaneously. Consistent with the existence of separate receptor sites for phenylalkylamines versus DHPs and benz(othi)azepines, permanently charged DHPs and benz(othi)azepines approach their receptor sites from the extracellular side, whereas charged phenylalkylamines approach their receptor site from the intracellular side (19–22, 56–58). The close proximity of the receptor sites for DHPs, phenylalkylamines, and benz(othi)azepines (37, 38, 53–55) raises the possibility that individual amino acid residues may be required for high affinity binding of more than one of these ligands. In agreement with that expectation, amino acid residues required for high affinity binding of two or more classes of these drugs have been identified (e.g. Tyr\(^{1463}\), Ala\(^{1467}\), and Ile\(^{1471}\); Refs. 37, 38, and 53–55 and this study). In fact, Tyr\(^{1463}\) is essential for high affinity binding of all three classes of Ca\(^{2+}\) channel antagonists. How can these findings be accommodated with earlier results indicating separate binding sites for phenylalkylamines, benz(othi)azepines, and DHPs? Phenylalkylamines are considered to block Ca\(^{2+}\) channels directly by occluding the transmembrane pore through which Ca\(^{2+}\) ions move. In contrast, DHPs are thought to bind to identical or overlapping sites at which agonists increase Ca\(^{2+}\) channel activity and antagonists reduce Ca\(^{2+}\) channel activity, so they cannot bind in a manner that blocks the pore. Therefore, it is our working hypothesis that these different classes of drugs bind to closely spaced sites that have both shared and distinct molecular components and have different spatial relationships to the pore. Binding of DHPs affects pore function allosterically, whereas binding of phenylalkylamines blocks the pore directly. The relationships among the amino acid residues that comprise these receptor sites are defined in more detail in the following paper (59).

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**Fig. 5.** The domain interface model of the DHP receptor site. The amino acid sequences of transmembrane domains IIIS6 and IVS6 are illustrated as a helix. White letters inside black circles represent amino acids that when mutated have a reduction in DHP binding that is greater than 5-fold. Black letters inside shaded circles represent amino acids that when mutated have a significant reduction in DHP binding that is less than 5-fold. The dotted circle represents Phe\(^{1462}\), which when changed to alanine has 416-fold increased affinity for block by DHPs. Gray letters inside white circles are amino acids that had no effect on DHP binding when mutated. Arrows point to residues that have been shown to be critical for block by phenylalkylamines and benz(o)-thiapinezepines (53–55). A schematized DHP ligand is depicted contacting the key binding determinants in the two transmembrane segments.
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