Molecular Determinants of High Affinity Dihydropyridine Binding in L-type Calcium Channels*

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The pore-forming \( \alpha \) subunit of L-type voltage-gated \( \text{Ca}^{2+} \) channels is pharmacologically modulated by dihydropyridine (DHP) \( \text{Ca}^{2+} \) antagonists and agonists. Site-directed mutation of amino acids within transmembrane segments IIIS6 and IVS6 to those characteristic of DHP-insensitive channels revealed 2 mutations in IIIS6 (Y1048F and Y1052F) and 4 mutations in IVS6 (Y1365I, M1366F, I1372M, and I1373L) with increased \( K_D \) values for \( (+)\text{[H]}\text{PN200-110} \) binding. A tyrosine residue (Y1048) in IIIS6 that is conserved between DHP-sensitive and -insensitive \( \text{Ca}^{2+} \) channels was also altered by mutagenesis. Y1048F had a \( K_D \) for \( (+)\text{[H]}\text{PN200-110} \) binding that was increased 12-fold, and Y1048A had a \( K_D \) at least 1000-fold higher than that of wild-type. These results support the hypothesis that transmembrane segments IIIS6 and IVS6 both contribute critical amino acid residues to the DHP receptor site and that Tyr-1048 within transmembrane segment IIIS6 is required for high affinity DHP binding, even though it is conserved between DHP-sensitive and -insensitive \( \text{Ca}^{2+} \) channels.

L-type voltage-gated \( \text{Ca}^{2+} \) channels are pharmacologically modulated by dihydropyridine (DHP) \( \text{Ca}^{2+} \) antagonists and agonists, while non-L-type \( \text{Ca}^{2+} \) channels are insensitive to DHPs. Peptide segments which contribute to the DHP receptor site have been localized by photoaffinity labeling and antibody mapping to transmembrane segments IIIS6 and IVS6 and adjacent extracellular segments of the \( \alpha \) subunit (1–3). Charged DHPs can only reach their receptor site from the extracellular side of the membrane (4). Access is optimal when the length of the alkyl spacer chain between the charged moiety and the binding center of the ligand is 10 methylene groups, suggesting that the DHP receptor site is approximately 11–14 Å into the lipid bilayer (5). Together, these results suggest the DHP receptor site is located within transmembrane segments IIIS6 and IVS6 about 25–35% of the distance across the lipid bilayer. In addition, a site in the intracellular carboxyl-terminal domain has been photoaffinity-labeled by photoreactive DHPs (6). Like DHP antagonists, DHP agonists also act from the extracellular side of the membrane (7). Analysis of chimeric \( \text{Ca}^{2+} \) channels (8) showed that the extracellular end of segment IVS6 is important for the action of DHP agonists. Here, we use site-directed mutagenesis and radioligand binding to identify individual amino acids in transmembrane segments IIIS6 and IVS6 that are critical determinants of DHP binding.

EXPERIMENTAL PROCEDURES

Materials—tsA-201 cells were provided by Dr. Robert DuBridge (Cell Genesys, Foster City, CA). cDNA encoding the \( \alpha_1 \) (9, 10) and \( \alpha_2 \delta \) (10) subunits cloned from 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).

Construction and Expression of Mutant \( \text{Ca}^{2+} \) Channels—The construction of wild-type and mutant expression plasmids encoding the entire \( \alpha_1 \) subunit of the voltage-gated \( \text{Ca}^{2+} \) channel from rabbit skeletal muscle was accomplished as described previously (11) with cDNA encoding wild-type or mutant \( \alpha_1 \) subunit expression plasmids and expression plasmids carrying cDNA encoding the \( \beta_1 \) and \( \alpha_2 \delta \) subunits of rabbit skeletal muscle \( \text{Ca}^{2+} \) channels such that the molar ratio of the plasmids was 1:1:1. Cells were harvested 20–40 h following transfection, and membranes were prepared by differential centrifugation and analyzed as described previously (11).

Radioligand Binding—Equilibrium binding assays were performed in Buffer A (50 mM Tris, 1 mM \( \text{Ca}^{2+} \), 100 mM phenylmethylsulfonyl fluoride, 1 \( \mu \)M pepstatin A, 100 \( \mu \)M benzamidine, pH 8.0) with 0–20 mg of membrane protein and 0.1–20 mM \( (+)\text{[H]}\text{PN200-110} \) at 22 °C for 90–120 min. Nonspecific binding was determined in the presence of 1 \( \mu \)M \( (+)\text{[H]}\text{PN200-110} \) and the bound ligand was 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 \( (K_D) \) were determined using the program LIGAND (12).

Competition experiments between \( (+)\text{[H]}\text{PN200-110} \) and \( (+)\text{[H]}\text{Bay K 8644} \) were performed in Buffer A using 20–200 mg of membrane protein, concentrations of \( (+)\text{[H]}\text{PN200-110} \) equal to the \( K_D \) for \( (+)\text{[H]}\text{PN200-110} \) for the wild-type or mutant channel under study (determined by Scatchard analysis in the presence of 1 mM free \( \text{Ca}^{2+} \)), and the indicated concentration of DHP agonist \( (+)\text{[H]}\text{Bay K 8644} \) at 22 °C for 90–120 min. Under these conditions, approximately 50% of the receptor sites were occupied by \( (+)\text{[H]}\text{PN200-110} \) in the absence of the competitor, and the concentration of \( (+)\text{[H]}\text{Bay K 8644} \) required to reduce this level of binding by 50% \( (IC_{50}) \) was related to the dissociation constant \( (K_D) \) of \( (+)\text{[H]}\text{Bay K 8644} \) using the method of Cheng and Prusoff (13).

Immunoblot Analysis—Assessment of expression of \( \alpha_1 \) subunit protein was carried out by Western blot analysis. 0.4 mg of membrane protein was dissolved in 50 mM Tris, 3% SDS, 1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol, agitated for 30 min at 70 °C, electrophoresed on a 6% SDS-polyacrylamide gel, and electroblotted to nitrocellulose for 12–16 h at 4 °C. After blocking with 0.1% skim milk powder in TBS (50 mM Tris, 0.15 mM NaCl, pH 7.4), the nitrocellulose was incubated with antipeptide antibodies against \( \alpha_1 \). Following washing in TBS, the bound antibodies were visualized using horseradish peroxidase-linked protein A and chemiluminescent detection (Amersham).

RESULTS AND DISCUSSION

Transmembrane Segments IIIS6 and IVS6 Are Important Determinants of High Affinity DHP Binding—Experiments using permanently charged or photoreactive DHP antagonists suggest that the extracellular portion of transmembrane segments IIIS6 and IVS6 form the core of the DHP receptor site (1–5). In order to identify individual amino acids that are critical determinants of DHP binding, site-directed mutagenesis was used to alter amino acid residues within the extracel-

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1 The abbreviation used is: DHP, dihydropyridine.
lular portions of IIIS6 and IVS6 and the entire loop regions connecting them (Fig. 1A). These regions contain more than 200 amino acid residues. Therefore, we took advantage of differences in DHP sensitivity of L-type and non-L-type Ca\(^{2+}\) channels by substituting the amino acids present in the DHP-insensitive channels into the corresponding positions of the DHP-sensitive skeletal muscle L-type channel. This strategy reduced the number of amino acids analyzed from greater than 200 to 75. DHP binding affinity was monitored by measurements of saturation binding using the radiolabeled DHP antagonist (+)-[\(^{3}H\)PN200-110 and Scatchard analysis. A high ratio of specific to nonspecific binding was observed in binding experiments with both wild-type Ca\(^{2+}\) channels (not shown) and with mutant channels having substantially reduced affinity (e.g. mutant m1362-73, Fig. 1B, left). The linearity of the Scatchard plot (Fig. 1B, right) indicates a single class of high affinity binding sites with a \(K_D\) of 0.27 nM for wild-type and 5.74 nM for the mutant (Fig. 1B, right). Equilibrium dissociation constants were measured accurately for mutants with \(K_D\) values up to 21 times higher than wild-type.

The 75 target amino acid residues were screened initially in clusters such that each mutant channel contained alterations in 1 to 11 nearby amino acids (Fig. 1, C and D). For example, mutant m956-74 contained 10 amino acid substitutions that converted amino acids in the segment containing residues 956 to 974 from their identity in DHP-sensitive Ca\(^{2+}\) channels (Fig. 1C, top line) to the corresponding amino acid residue in DHP-insensitive Ca\(^{2+}\) channels (Fig. 1C, second line). The \(K_D\) values determined for wild-type and mutant Ca\(^{2+}\) channels are illustrated as bar graphs in Fig. 1C (right) for domain III mutations and in Fig. 1D (right) for domain IV mutations. Only mutants m956-74, Y1021K, and m1045-53 in domain III and m1362-73 in domain IV have \(K_D\) values for (+)-[\(^{3}H\)PN200-110 binding that are significantly higher than wild-type. The largest effect in domain III was observed for mutant m1045-53 in which substitution of 8 amino acid residues increased the \(K_D\) value 7.2-fold. The largest effect overall was observed for mutant m1362-73 in which the 6 altered amino acids in the IVS6 transmembrane segment resulted in a 21-fold higher \(K_D\) for (+)-[\(^{3}H\)PN200-110 binding (Fig. 1B). The combined effects of mutations m956-74, Y1021K, m1045-53, and m1362-73 predict a 4.1 kcal/mol increase in the free energy for binding (\(\Delta G\)), which corresponds to more than a 1000-fold decrease in the binding affinity compared with wild-type. These results are in good agreement with experiments using photoactive DHPs which suggest that the DHP receptor site is located within and adjacent to the S6 segments in domains III and IV have substantially reduced affinity. The identities of the eight individual amino acid changes in m956-74, Y1021K, m1045-53, and m1362-73 in domain III and m1362-73 in domain IV (shaded) and the connecting loops between segments S5 and S6 (bold) were analyzed by site-directed mutagenesis and radioligand binding. B, left, equilibrium binding on m1362-73 membranes showing the levels of total (inverted triangles), nonspecific (squares), and specific (circles) binding of the DHP antagonist (+)-[\(^{3}H\)PN200-110. Right, Scatchard transformation of equilibrium binding data for m1362-73 (circles) and wild-type (diamonds). Equilibrium binding experiments were done as described under "Experimental Procedures." C and D, amino acids corresponding to segments of the Ca\(^{2+}\) channel that span the S5-S6 connecting loops and portions of the S6 segments in domains III (C) and IV (D; see bold and shaded regions in A, above) are indicated for the wild-type and the altered \(K_D\) value. These mutations are indicated below the corresponding wild-type sequence. The name of each mutant is indicated to the left (bold), and the numbers correspond to the amino acid positions spanned by each cluster of mutations. Shaded regions correspond to amino acids within transmembrane segments. The conserved Ca\(^{2+}\)-binding glutamate residues that are critical for ion selectivity and permeation, Glu-1014 and Glu-1323, are indicated by e (see also A). Dissociation constants derived from Scatchard transformation of equilibrium binding data (see "Experimental Procedures") are indicated by horizontal bar plots to the right. Each experiment was repeated at least three times, and data are means ± S.E.

![Figure 1](image)

**FIG. 1.** Effects of mutation of clusters of amino acid residues within and adjacent to the S6 transmembrane segments in domains III and IV on high-affinity DHP binding. A, proposed transmembrane topology of domains III and IV of the \(\alpha_{1C}\) subunit of voltage-gated Ca\(^{2+}\) channels. Each domain consists of six transmembrane segments and the loops connecting segments S5 and S6 of each domain are thought to contain the selectivity filter of the channel. The relative positions of Glu-1014 and Glu-1323 are indicated by e. Amino acids that correspond to portions of the sixth transmembrane segments of domains III and IV (shaded) and the connecting loops between segments S5 and S6 (bold) were analyzed by site-directed mutagenesis and radioligand binding. B, left, equilibrium binding on m1362-73 membranes showing the levels of total (inverted triangles), nonspecific (squares), and specific (circles) binding of the DHP antagonist (+)-[\(^{3}H\)PN200-110. Right, Scatchard transformation of equilibrium binding data for m1362-73 (circles) and wild-type (diamonds). Equilibrium binding experiments were done as described under "Experimental Procedures." C and D, amino acids corresponding to segments of the Ca\(^{2+}\) channel that span the S5-S6 connecting loops and portions of the S6 segments in domains III (C) and IV (D; see bold and shaded regions in A, above) are indicated for the wild-type and the altered \(K_D\) value. These mutations are indicated below the corresponding wild-type sequence. The name of each mutant is indicated to the left (bold), and the numbers correspond to the amino acid positions spanned by each cluster of mutations. Shaded regions correspond to amino acids within transmembrane segments. The conserved Ca\(^{2+}\)-binding glutamate residues that are critical for ion selectivity and permeation, Glu-1014 and Glu-1323, are indicated by e (see also A). Dissociation constants derived from Scatchard transformation of equilibrium binding data (see "Experimental Procedures") are indicated by horizontal bar plots to the right. Each experiment was repeated at least three times, and data are means ± S.E.

these amino acids to the binding energy are not independent. A Conserved Amino Acid Residue Required for High Affinity DHP Binding—The altered amino acids in m1045-53 flank a Tyr residue (Tyr-1048) that is conserved between DHP-sensitive and -insensitive Ca\(^{2+}\) channels and is positioned at the same predicted distance into the lipid bilayer (25–35%) as Y1365, a critical residue in segment IVS6. In order to examine the importance of Tyr-1048 and Tyr-1365 in more detail, these amino acids were altered within these segments, and these mutations are indicated below the corresponding wild-type sequence. The name of each mutant is indicated to the left (bold), and the numbers correspond to the amino acid positions spanned by each cluster of mutations. Shaded regions correspond to amino acids within transmembrane segments. The conserved Ca\(^{2+}\)-binding glutamate residues that are critical for ion selectivity and permeation, Glu-1014 and Glu-1323, are indicated by e (see also A). Dissociation constants derived from Scatchard transformation of equilibrium binding data (see "Experimental Procedures") are indicated by horizontal bar plots to the right. Each experiment was repeated at least three times, and data are means ± S.E.

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**Molecular Determinants of Dihydropyridine Binding**

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**Domain III Domain IV**

![Diagram](image)

**A**

![Bar Graph](image)

**B**

![Graph](image)

**C**

![Bar Graph](image)

**D**

![Graph](image)
binding. The isosteric alterations made in Y1048F and Y1365F should cause minimal change in protein structure (14), so the ΔΔG values of 1.5 and 0.7 kcal/mol, respectively, are likely to reflect changes in interaction of the bound DHP with these residues. Mutations Y1048A and Y1365A have larger effects on DHP binding. The Kd for binding in Y1365A is increased by 6.1-fold. Y1048A exhibits no detectable DHP binding at concentrations of (+)-[3H]PN200-110 up to 25 nM (Fig. 2C), but immunoblots indicate that the protein is expressed well (Fig. 2D). Failure to observe specific binding at 25 nM ligand concentration indicates that the Kd is at least 250 nM. Thus, the alteration of a single amino acid in the mutant Y1048A decreases the affinity of the channel for (+)-[3H]PN200-110 by more than 1000-fold. The magnitude of this effect suggests strongly that Tyr-1048 interacts directly with bound DHPs. Tyr-1048 may be the amino acid residue that reacted with the photoactive benzofurazane group of (+)-[3H]PN200-110 in the previous photoaffinity labeling experiments that identified III56 as forming the core of the DHP receptor site (1). It is of interest that the most critical amino acid residue identified in this study is present in both DHP-sensitive and -insensitive channel types. Evidently, conserved amino acid residues in segment III56 may be essential for formation of the high affinity DHP binding site in addition to residues that are unique to the DHP-sensitive channels.

Effects of Mutations in Transmembrane Segments III56 and IV56 on Binding of DHP Agonists—Competition assays were used to determine whether the same amino acids that determine DHP antagonist sensitivity also determine the affinity of the channel for DHP agonists. Each of the mutations which reduce affinity for DHP antagonists also reduces the affinity for the DHP agonist (+)-Bay K 8644 (Fig. 3A). For each of the mutants in domains III and IV, the increase in Kd for the agonist (+)-Bay K 8644 (Kd(mut)/Kd(wt)) is comparable to that for the antagonist (+)-[3H]PN200-110 (Kd(mut)/Kd(wt)) (Fig. 3B). These results demonstrate that the mutation of amino acids that determine the sensitivity of the channel to DHP antagonists also affects the sensitivity of the channel to DHP agonists to a similar degree. The binding of non-DHP agonists such as the benzoylpyrrole FPL 64176 (15) is not affected by these mutations, indicating that these amino acids specifically affect the binding of the DHP class of agonists rather than causing loss of affinity for all channel activators. Thus, there are many common determinants of the binding affinity of DHP agonists and antagonists, consistent with the conclusion that these two classes of channel modulators bind to the same receptor site. The distinctive properties of the DHP agonists are likely to be conferred by induction of a specific conformation of the single DHP receptor site due to the stereochromeny of interaction with the agonists, rather than by interaction with a different receptor site.

In contrast to our results, Tang et al. (8) found that activation of cardiac L-type Ca2+ channels by DHP agonists measured electrophysiologically was strongly affected in a chimeric channel (CBC7, Ref. 8) in which 1 of the 24 changes was analogous to Y1365I, but block by antagonists was unaffected. Two potentially important differences between the two studies—may have caused the apparent discrepancy. First, Tang et al. (8) measured the effect of agonist and antagonist binding rather than the binding interaction itself. Therefore, it is possible that the 24 changes in amino acid residues within and near transmembrane segment IV56 in chimera CBC7 caused structural changes that altered the coupling of DHP binding to activation and inhibition of the Ca2+ channel, respectively, such that inhibition is favored over activation. In fact, the efficacy of channel activation at saturating concentrations of agonist was reduced 4.1-fold in their experiments (Fig. 5B of Ref. 8), providing direct experimental evidence for an allosteric effect that made activation of the CBC7 mutant by DHPs unfavorable. Such allosteric changes would favor the binding of antagonists and could therefore compensate for the large decrease in the intrinsic binding affinity for DHP agonists that we have observed for the Y1365I mutation. Second, Tang et al. (8) measured the effects of DHP antagonists at the resting membrane potential where antagonist binding affinity is about 300-fold lower than in our work. Therefore, it is possible that
the amino acid residues in segment IVS6 that we have identified as components of the high affinity antagonist binding site do not interact as strongly with antagonists in the low affinity state present at the resting membrane potential as they do with the high affinity state studied in this work. If this is correct, these residues would be implicated as determinants of the state-dependent affinity for DHP antagonists. In any case, our results show that Y1365, I1372, and I1373 in segment IVS6 are required for high affinity binding of both DHP agonists and antagonists in agreement with previous photoaffinity labeling results (1, 2, 16).

Location of the Critical Amino Acid Residues for High Affinity DHP Binding—Previous results demonstrating specific photoaffinity labeling of the IIIS6 and IVS6 transmembrane segments by three different photoaffinity reagents (1-3) indicate that DHP antagonists interact directly with amino acid residues within these regions. Our experiments identify specific amino acid residues within these two transmembrane segments which are required for high affinity binding of both agonist and antagonist DHPs. Together, these results support the hypothesis (1, 16) that the amino acid residues in transmembrane segment IIIS6, including Tyr-1048, form the core of the DHP receptor site because this segment was specifically labeled by PN200-110, an intrinsically photoreactive dihydropyridine (1), and mutation of these residues has the largest effect on high affinity DHP binding. Photoaffinity labeling studies with azidopine and diazepine, each of which has a photoreactive group on a long arm, also resulted in incorporation of photolabel into the extracellular segment preceding transmembrane segment IIIS6 (1, 2), and it was concluded that this represented labeling of a peripheral region of the DHP receptor site (1, 16). Consistent with this interpretation, mutation of amino acid residues in this segment of the α, subunit did not result in large reductions in DHP affinity (Fig. 1).

A previous study of chimeric Ca2+ channels concluded that the “SS2-S6 region of motif III” of the α, subunit is not required for DHP action (8). However, in that study, none of the amino acid residues in transmembrane segment IIIS6 which have been identified as components of the DHP binding site in our experiments were mutated (see description of construction of chimera CBC3 under “Experimental Procedures” of Ref. 8, p. 1020). Moreover, the critical Tyr-1048 could not have been identified by the chimeric approach in any case because it is conserved between DHP-sensitive and non-DHP-sensitive Ca2+ channels. In further support of the importance of domain III in DHP binding, recent studies implicate the nearby IIIS2-IIIS3 segment in the voltage dependence of DHP binding, but suggest that it may not be involved directly in formation of the DHP receptor site (17).

The amino acid residues in transmembrane segments IIIS6 and IVS6 which are required for high affinity binding of DHP antagonists are located in nearly analogous positions in the two putative α helices (Fig. 4). These results are consistent with the domain-interface model of DHP binding (1, 16), which suggests that DHPs bind between the corresponding faces of these two transmembrane segments in domains III and IV. Evidently, DHP binding at the interface between these transmembrane segments in domains III and IV modulates channel gating. Nearby amino acid residues in transmembrane segment IVS6 are required for high affinity binding of the phenylalkylamine desmethoxyverapamil (18), providing a molecular basis for the allosteric interactions between the phenylalkylamines and DHPs in binding to Ca2+ channels.

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