Molecular Determinants of High Affinity Phenylalkylamine Block of L-type Calcium Channels in Transmembrane Segment IIIS6 and the Pore Region of the α_{1} Subunit

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Recent studies of the phenylalkylamine binding site in the α_{1C} subunit of L-type Ca^{2+} channels have revealed three amino acid residues in transmembrane segment IVS6 that are critical for high affinity block and are unique to L-type channels. We have extended this analysis of the phenylalkylamine binding site to amino acid residues in transmembrane segment IIIS6 and the pore region. Twenty-two consecutive amino acid residues in segment IIIS6 were mutated to alanine and the conserved Glu residues in the pore region of each homologous domain were mutated to Gln. Mutant channels were expressed in tsA-201 cells along with the β_{1b} and α_{2δ} auxiliary subunits. Assay for block of Ba^{2+} current by dihydropyridines and benzothiazepines revealed that mutation of five amino acid residues in segment IIIS6 and the pore region that are conserved between l-type and non-l-type channels (Glu{1}^{1152}, Phe{1}^{1164}, Val{1}^{1165}, Glu{1}^{1168}, and Glu{1}^{1419}) and one l-type-specific amino acid (Ile{1}^{1153}) decreased affinity for (−)-D888 from 10–20-fold. Combination of the four mutations in segment IIIS6 increased the IC_{50} for block by (−)-D888 to approximately 9 μM, similar to the affinity of non-l-type Ca^{2+} channels for this drug. These results indicate that there are important determinants of phenylalkylamine binding in both the S6 segments and the pore regions of domains III and IV, some of which are conserved across the different classes of voltage-gated Ca^{2+} channels. A model of the phenylalkylamine receptor site at the interface between domains III and IV of the α_{1} subunit is presented.

L-type Ca^{2+} channels are found in many excitable cell types, including muscle, neuronal, and endocrine cells, where they initiate Ca^{2+}-dependent responses such as contraction and secretion (reviewed in Refs. 1 and 2). The pore forming α_{1} subunits of voltage-gated Ca^{2+} channels consist of four homologous domains (I-IV), each containing six putative transmembrane segments (S1-S6) (3). The role of l-type channels in initiating muscle contraction within the cardiovascular system has made them important therapeutic targets for the treatment of hypertension, angina pectoris, and cardiac arrhythmia. Three major classes of l-type Ca^{2+} channel block-

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Glu\textsuperscript{18760}, Glu\textsuperscript{709}, Glu\textsuperscript{1118}, and Glu\textsuperscript{1419} (20), to Gln and screened the resulting mutant channels for sensitivity to (--)D888. We report here that Glu\textsuperscript{1118} and Glu\textsuperscript{1419} in the putative pore-lining segments of homologous domains III and IV, respectively, are also involved in high affinity block of \( \text{t-type Ca}\textsuperscript{2+} \) channels by phenylalkylamines.

**EXPERIMENTAL PROCEDURES**

**Construction of Mutants**—All mutations were constructed using oligonucleotide-directed mutagenesis as described previously (21). The IIIS6, E1118Q, and E1419Q mutations were inserted into full-length \( \alpha_1 \) subunit constructs in the expression vector Zenn229 (Dr. Eileen Mulvihill, Zymogenetics Corp., Seattle) using the 1.5-kilobase SpeI/DraIII fragment and the 272-base pair DraI/III fragment in a three-way ligation. The E363Q mutation was inserted into the full-length \( \alpha_1 \) subunit construct using the 1.4-kilobase NcoMI/BglII fragment. The E709Q mutation was inserted into the full-length \( \alpha_1 \) subunit construct using the 1.3-kilobase Sau3AI/BglII fragment and the 272-base pair DraI/III fragment. All mutations were confirmed by cDNA sequencing.

**Cell Culture**—tsA201 cells, a subclone of the human embryonic kidney cell line HEK293 that expresses SV40 T antigen (a gift of Dr. Robert Dubridge, Cell Genesis, Foster City, CA), were maintained in monolayer culture in Dulbecco’s modified Eagle’s medium/Ham’s F-12 medium (Life Technologies, Inc., supplemented with 10% fetal bovine serum (HyClone, Logan, UT), and incubated at 37 °C in 10% \( \text{CO}_2 \).

**Expression**—Wild-type and mutant \( \alpha_1 \) channel subunits (22) were expressed with \( \beta_2(23) \) and \( \alpha_\delta(24) \) channel subunits and CD2 antisera (EBO-pcd-Leu2, American Type Culture Collection) in tsA-201 cells (derived from HEK 293 cells) by transient CaPO\(_4\) transfection as described (25). Transfectants were selected by labeling with anti-CD8 antibodies conjugated to latex beads (Dynal A.S., Oslo, Norway).

**Electrophysiology**—(--)D888 was applied to cells recorded in the whole cell patch-clamp configuration by the addition of 0.2 ml of a 6 \text{ mM} MgCl\(_2\) saline, using an on-line P/4 subtraction paradigm.

**RESULTS**

**Block of Wild Type Ca\textsuperscript{2+} Channels by (--)D888**—The \( \text{t-type Ca}\textsuperscript{2+} \) channel \( \alpha_1 \) subunit (22) was expressed in tsA-201 cells (25) together with the \( \beta_2(23) \) and \( \alpha_\delta(24) \) subunits. Ba\textsuperscript{2+} currents through the resulting \( \text{t-type Ca}\textsuperscript{2+} \) channels were blocked by (--)D888; a concentration of 50 \text{ mM} (--)D888 reduced the Ba\textsuperscript{2+} current by approximately 50% (Fig. 1A). The block by (--)D888 was rapid and reached equilibrium within 200 s (14). Analysis of equilibrium block of Ba\textsuperscript{2+} currents by a range of concentrations of (--)D888 yielded an IC\textsubscript{50} of 48 ± 5 \text{ nM} (Fig. 1F).

**Effects of Mutations in Transmembrane Segment IIIIS6 of the \( \alpha_1 \) Subunit**—The putative transmembrane segment IIIIS6 of the \( \alpha_1 \) subunit of \( \text{t-type Ca}\textsuperscript{2+} \) channels contains primarily hydrophobic amino acid residues.

\[ \alpha_1 \text{IIIIS6} = \text{ISIFIIYIIIIIAFFMMNIFVGFVI} \] 1169

\[ \alpha_1 \text{IIIIS6} = \text{L---------------YVV-FVVF-FV---------------ALI-} \] 1373

**Scheme 1**

Of the 25 amino acid residues predicted to comprise this transmembrane segment, 14 are different in the phenylalkylamine-insensitive (14) \( \alpha_1 \) subunits of N-type Ca\textsuperscript{2+} channels (Scheme 1). To assess the role of the individual amino acid residues in the IIIIS6 segment of \( \alpha_1 \) in high affinity block by (--)D888, we mutated Ala\textsuperscript{1157} to Pro as in N-type Ca\textsuperscript{2+} channels (Scheme 1), mutated the other amino acids in IIIIS6 to Ala, and screened the mutant channels for sensitivity to (--)D888. Ala was chosen for substitution because it has minimal effects on protein secondary structure when substituted in a helix in the core of proteins (26) and therefore is expected to reduce the hydrophobicity and size of the amino acid residue in each position in this putative \( \alpha \) helix without causing global conformational change.

All of the mutant \( \alpha_1 \) subunits studied formed functional Ca\textsuperscript{2+} channels in tsA-201 cells except A1157P. Seventeen of these single amino acid mutations to Ala studied, only I1153A (IC\textsubscript{50} = 48 ± 5 \text{ nM}) caused significant increases in the IC\textsubscript{50} for block of Ba\textsuperscript{2+} currents by (--)D888. For example, the mutation F1164A caused a large increase in the concentration of (--)D888 required for block of the Ba\textsuperscript{2+} current (Fig. 1C) and approximately a 10-fold shift to higher concentration of the inhibition curve for (--)D888 (Fig. 1F). The IC\textsubscript{50} values for block of Ba\textsuperscript{2+} currents through the wild type and all of the mutant \( \alpha_1 \) subunits are illustrated as a bar graph in Fig. 2. Of the 21 amino acid mutations to Ala studied, only I1153A (IC\textsubscript{50} = 601 ± 106 \text{ nM}) caused significant increases in the IC\textsubscript{50} for...
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Functional Properties of IIIS6 Mutants—To examine the specificity of the effects of the IIIS6 mutations on Ca\(^{2+}\) channel function, we compared their kinetic and voltage-dependent properties with those of Ca\(^{2+}\) channels containing wild type a\(_{\text{IIIs6}}\). Current-voltage relationships were generally similar for wild type and for the mutant a\(_{\text{IIIs6}}\) subunits having altered affinity for (-)-D888 (Fig. 3, A and B) with peak Ba\(^{2+}\) currents observed at +10 to +20 mV in each case. However, closer analysis of conductance-voltage relationships revealed small but significant differences (wild type, V\(_{\text{rev}}\) = +11.5 mV; Y1152F, V\(_{\text{rev}}\) = +6 mV; I1153A, V\(_{\text{rev}}\) = +3.7 mV; F1164A, V\(_{\text{rev}}\) = +6.8 mV; and V1165A, V\(_{\text{rev}}\) = +4.0 mV). These voltage shifts were not due to differences in the time from forming the whole cell patch clamp configuration because current-voltage relations were first measured at 5 min after break-in, and no additional shifts were observed after that time. Although the voltage dependence of activation was significantly different for the single mutations, the voltage dependence of channel activation was not significantly different in the combination mutant YIFV compared with wild type (YIFV, V\(_{\text{rev}}\) = +14.3 mV), suggesting that the effects of the single mutations were compensated in the combined mutant.

The IIIS6 mutations also affected the apparent reversal potential (E\(_{\text{rev}}\)) of the mutant channels, which is a measure of their ion selectivity. Although the E\(_{\text{rev}}\) of the mutant YIFV was shifted only slightly from wild type (YIFV, E\(_{\text{rev}}\) = +56.3 ± 2.9 mV, n = 6; wild type, E\(_{\text{rev}}\) = +61.3 mV ± 4.4, n = 10), both single Tyr\(^{1152}\) mutations were shifted approximately 15 mV (Y1152A, E\(_{\text{rev}}\) = +46.9 ± 6.1 mV, n = 4; Y1152F, E\(_{\text{rev}}\) = +46.9 ± 3.1 mV, n = 4). The E\(_{\text{rev}}\) values of mutants I1153A and V1165A were also substantially shifted from wild type (I1153A, E\(_{\text{rev}}\) = 47.4 ± 1.8 mV, n = 5; V1165A, E\(_{\text{rev}}\) = 42.2 ± 2.3 mV, n = 5), whereas the E\(_{\text{rev}}\) of mutant F1164A was more moderately affected (F1164A, E\(_{\text{rev}}\) = 53.1 ± 3.6 mV, n = 5). These results are consistent with the idea that the IIIS6 segment contributes to the lining of the pore of Ca\(^{2+}\) channels, and mutations of amino acid residues in this segment therefore alter the selectivity of ion conductance.

In contrast to their lack of effect on channel activation, phenylalkylamines cause Ca\(^{2+}\) channel inactivation curves to shift in the hyperpolarizing direction, indicating that block by these compounds is more potent at depolarized potentials where inactivation is favored (27–29). Therefore, mutations that alter voltage-dependent inactivation of Ca\(^{2+}\) channels may affect binding and block by phenylalkylamines indirectly. To avoid such effects, all of our experiments were carried out at a holding potential of ~60 mV, considerably more negative than steady-state inactivation of l-type Ca\(^{2+}\) channels, so further reduction in voltage-dependent inactivation by mutations should not significantly reduce affinity for (-)-D888. Nevertheless, we have characterized the voltage dependence of inactivation of the mutant Ca\(^{2+}\) channels in detail (Fig. 3C). Muta-
tion of residues Ile1153, Phe1164, and Val1165 caused positive shifts in $V_{1/2}$ for inactivation ($V_{1/2} = -14.5 \text{ mV}$; I1153A, $V_{1/2} = -11.6 \text{ mV}$; and F1164A, $V_{1/2} = -9.1 \text{ mV}$) compared with wild type ($V_{1/2} = -17.7 \text{ mV}$). Removal of the hydroxyl group from Tyr1152 did not affect the voltage dependence of steady-state inactivation significantly ($V_{1/2} = -18.8 \text{ mV}$), but removal of the aromatic ring from that position in IIIS6 resulted in a negative shift of approximately 11 mV in $V_{1/2}$ ($Y1152A, V_{1/2} = -29.0 \text{ mV}$). Inactivation curves for the combination mutant YIFV are approximately 12 mV more positive than wild type ($V_{1/2} = -5.7 \text{ mV}$).

To examine the correlation between shifts in the voltage dependence of inactivation and the affinity for block by $(-)$-D888 quantitatively, we plotted the IC$_{50}$ values for the IIIS6 mutant channels against their half-inactivation voltage (Fig. 3A). $IC_{50}$ values (Fig. 3D). Thus, because inactivation is minimal at the holding potential used in these experiments (−60 mV) and no correlation of $IC_{50}$ with $V_{1/2}$ is observed, the decrease in $(-)$-D888 affinity cannot be ascribed to changes in the intrinsic voltage dependence of channel inactivation in the mutants. On the other hand, the correlation plot of Fig. 3D may reveal the reason for the increase in affinity caused by mutation Y1152A. This mutant has by far the most negative $V_{1/2}$ value (−29 mV) and also has the highest affinity for $(-)$-D888. Enhanced steady-state inactivation at −60 mV for this mutant may contribute substantially to its increased affinity for $(-)$-D888.

**Effects of Mutations in the Pore Region of the α $_1$ Subunit—**

The α$_1$ subunits of voltage-gated Ca$^{2+}$ channels contain four highly conserved P-loops between the S5 and S6 transmembrane segments of each homologous domain that together form the selectivity filter through which the channel conducts cations. The high selectivity of voltage-gated Ca$^{2+}$ channels for divalent cations over monovalent ions and for Ca$^{2+}$ ions over other divalent ions is mediated by four Glu residues, one in each homologous domain, that are conserved across all Ca$^{2+}$ channels (20, 30). Because phenylalkylamines are thought to block ion channels by binding in the pore and the protonated amino group of $(-)$-D888 is positively charged at physiological pH and could potentially interact with the pore Glu residues through an electrostatic mechanism, we mutated each of these Glu residues individually to Gln to neutralize the negative charge that the acidic side chains have at physiological pH and tested the affinity for block by $(-)$-D888 in the four E→Q mutants. The mutant channels E363Q and E709Q having mutations in domains I and II, respectively, were both blocked by $(-)$-D888 with $IC_{50}$ values that were not significantly different from that of the wild type channel (Fig. 4, A and D). However, the mutant channels E1118Q and E1419Q in domains III and IV, respectively, both had significant increases in the $IC_{50}$ for $(-)$-D888 block (Fig. 4, B and D). The E1118Q mutant caused approximately a 20-fold rightward shift in the concentration

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**Fig. 3. Functional properties of IIIS6 mutant channels.** Current-voltage relationships in the absence of $(-)$-D888 for control and single amino acid mutations to Ala in IIIS6 (A) and Y1152A, Y1152F, and Y1152F, I1153A, F1164A, V1165A quadruple mutant (B). Mean values are shown ($n = 5$). Apparent reversal potentials were estimated by linear extrapolation of the data between +20 and +40 mV to the abscissa. C, voltage dependence of inactivation. Cells expressing the indicated Ca$^{2+}$ channels were depolarized from a holding potential of −60 mV to the indicated prepulse potentials for 5 s and then further depolarized to +10 mV for 100 ms to record Ba$^{2+}$ currents. Values for $V_{1/2}$ for inactivation were determined as described previously (14). D, relationship between $V_{1/2}$ for inactivation and $IC_{50}$ for $(-)$-D888, wt, wild type.
glutamate mutations on (−)-D888 block. Surprisingly, the presence of inactivation also varied widely. In mutant E363Q, the IC₅₀ for inactivation was not substantially different from wild type (E363Q Vₐ₅ = −16.3 mV), whereas Vₐ₅ values for inactivation of E709Q and E1419Q were shifted to more positive potentials by approximately 10 and 6 mV, respectively (E709Q, Vₐ₅ = −7.1 mV; E1419Q, Vₐ₅ = −11.6 mV). As with the mutations in segment III S6, there was no correlation between the effects of the mutations on IC₅₀ for (−)-D888 and Vₐ₅.

**DISCUSSION**

Contribution of Conserved and L-type-specific Amino Acid Residues in Transmembrane Segment IIIS6 to (−)-D888 Block of L-type Channels—As summarized in Fig. 2, we have analyzed the contribution of 21 single amino acid side chains in the transmembrane segment III S6 to block by (−)-D888. Of these four amino acids is unique to L-type channels (Ile₁₁₅₃), whereas the others are conserved across all types of voltage-gated Ca²⁺ channels (Scheme 1, underlined residues). The involvement of primarily conserved amino acid residues in segment III S6 in high affinity (−)-D888 binding stands in sharp contrast to the results of previous experiments in segment IV S6 where the only three amino acids required for (−)-D888 binding were unique to L-type channels (14–16). Our results show that conserved amino acid residues play crucial roles in the action of L-type selective drugs and demonstrate the importance of systematic analysis of all amino acid residues in putative drug binding sites in addition to chimeric approaches that target only isoform-specific residues.

The single amino acid mutation in this region with the largest effect was the conservative substitution of Phe for Tyr (Y₁₁₅₂F), in which only a single hydroxyl group was removed from the native channel structure. This result is similar to our previous observation (29) that mutation of a Tyr in IV S6...
\(\text{FIG. 5. A domain interface model for high affinity phenylalkylamine block.} \) A, amino acid residues in IVS6 that are unique to \(L\)-type channels as well as conserved amino acid residues from IISS6 and the pore region of domains III and IV converge to form the binding site for \((-\cdot\cdot\cdot\text{D888})\). The IISS6 and IVS6 residues could form a hydrophobic pocket that binds the aromatic moieties of the phenylalkylamines and stabilizes a charge interaction of the alkylamino group of the phenylalkylamines with Glu1118 and Glu1419. Black letters inside of black circles represent amino acids that, when mutated, disrupted \((-\cdot\cdot\cdot\text{D888})\) block. Gray letters in white circles represent amino acids that were not mutated. B, confluence of amino acids involved in dihydropyridine binding and phenylalkylamine block of \(L\)-type \(Ca^{2+}\) channels. The amino acid sequences of transmembrane domains IISS6 and IVS6 are shown as a helices. White letters inside of black symbols represent amino acids that, when mutated, disrupted binding/block. Black circles represent amino acids involved in \(DHP\) binding only. Black diamonds represent amino acids that disrupt \(PA\) block only. Black squares represent amino acids that disrupt both \(DHP\) binding and \(PA\) block. Black letters in white circles represent amino acids that, when mutated, did not affect \(DHP\) binding or \(PA\) block.

(Tyr\(^{1463}\)) to Phe caused a large reduction in sensitivity to \((-\cdot\cdot\cdot\text{D888})\). Simultaneous mutation of both Tyr\(^{1152}\) and Tyr\(^{1463}\) to Phe resulted in a channel with an approximately 100-fold increase in IC\(_{50}\) for \((-\cdot\cdot\cdot\text{D888})\) and normal inactivation properties.\(^1\) The importance of these two hydroxyl groups, which are potential hydrogen bond donors, suggests a privileged hydrogen bond between them and the single \(\text{meta}\)-methoxy group, a potential hydrogen bond acceptor, on the phenethylamide group of D888. This interaction is apparently not accessible for the lower affinity phenylalkylamines verapamil and D600 (methoxyverapamil), which possess an additional \(\text{para}\)-methoxy group (29).

Our results support the conclusion that the effects of the mutations of amino acid residues Tyr\(^{1152}\), Ile\(^{1153}\), Phe\(^{1164}\), and Val\(^{1165}\) on binding of \((-\cdot\cdot\cdot\text{D888})\) results from alteration of the interactions of the side chains of these residues with the bound drug. The effects of mutations of these residues are highly specific; mutations of adjacent residues to Ala have no effect on block by \((-\cdot\cdot\cdot\text{D888})\). There is no correlation between the effects of these mutations on activation or inactivation of \(Ca^{2+}\) channels and their effects on affinity for \((-\cdot\cdot\cdot\text{D888})\), indicating that the mutations do not cause their effects by indirect allosteric changes. Thus, our working hypothesis is that these four amino acid residues interact with \((-\cdot\cdot\cdot\text{D888})\) when it is bound to its receptor site on \(L\)-type \(Ca^{2+}\) channels with high affinity.

As suggested previously for amino acid residues in IVS6 that are required for high affinity phenylalkylamine block of \(L\)-type \(Ca^{2+}\) channels (14), it is likely that the IISS6 amino acid residues that affect \((-\cdot\cdot\cdot\text{D888})\) block also project into the ion-conducting pore. Like the IVS6 residue Tyr\(^{1463}\) mutation of Tyr\(^{1152}\), Ile\(^{1153}\), Phe\(^{1164}\), and Val\(^{1165}\) to Ala resulted in significant shifts in apparent reversal potential. As we showed for Y1463A (14), these shifts are likely to be due to increases in channel permeability to the normally impermeant organic cation \(N\)-methyl-D-glucamine, which is the principal cation in the intracellular solution. Thus, mutations of Tyr\(^{1152}\), Ile\(^{1153}\), Phe\(^{1164}\), and Val\(^{1165}\) change the shape or size of the ion-conducting pore.

**Contribution of Pore Glutamate Residues to \((-\cdot\cdot\cdot\text{D888})\) Block**—Phenylalkylamines are thought to bind in the pore of Ca\(^{2+}\) channels (17–19), and our results show that amino acid residues in transmembrane segments IISS6 and IVS6 that are involved in high affinity binding of \((-\cdot\cdot\cdot\text{D888})\) are also involved in maintaining the ion selectivity of the pore (Ref. 14 and this work). We therefore investigated the effects of mutating four highly conserved Glu residues, Glu\(^{363}\), Glu\(^{709}\), Glu\(^{1118}\), and Glu\(^{1419}\), in the pore region for two reasons. First, these acidic amino acid side chains apparently project into the pore to form the Ca\(^{2+}\) binding site(s) that confers Ca\(^{2+}\) selectivity to voltage-gated Ca\(^{2+}\) channels (20, 30). Second, these acidic amino acid side chains are negatively charged at physiological pH and potentially participate in electrostatic interactions with protonated (i.e., positively charged) phenylalkylamine molecules.

The spatial selectivity of the effects of mutations of these four pore Glu residues is consistent with the large body of data showing domains III and IV to be the site of binding of phenylalkylamines. Mutations of Glu\(^{1118}\) in domain III and Glu\(^{1419}\) in domain IV caused major reductions in the affinity for \((-\cdot\cdot\cdot\text{D888})\), whereas mutations of Glu\(^{363}\) in domain I and Glu\(^{709}\) in domain II had no appreciable effects. The decreased affinity of E1118Q and E1419Q for \((-\cdot\cdot\cdot\text{D888})\) was likely not caused by shifts in the voltage dependence of inactivation because E709Q, the pore mutant with the largest shift in steady-state inactivation, had no change in affinity for the drug. Although it is possible that the decreased affinity for \((-\cdot\cdot\cdot\text{D888})\) conferred by E1118Q and E1419Q is due to indirect effects, the specificity for these two mutations among the four pore Glu residues and their lack of correlated effects on channel gating argue that they interact directly with the bound drug molecule, most likely through

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electrostatic interactions with the positively charged amino group. This conclusion is consistent with previous studies showing that phenylalkylamines block ion channels in their positively charged, protonated state (18, 31) and with the evidence that phenylalkylamines bind within the pore of the Ca\(^{2+}\) channel.

(-)-D888 Binds to the Interface between Domains III and IV.—The results of this and previous studies (14–16) suggest that the receptor site for (-)-D888 is composed of at least four distinct subsites: IIIS6, the P-loop in the IIIS5-IIIS6 linker, IVS6, and the P-loop in the IVS5-IVS6 linker. The critical role of amino acid residues from both IIIS6 and IVS6 in (-)-D888 binding and block strongly suggest that these two transmembrane domains are juxtaposed to form a portion of the intracellular mouth of the ion-conducting pore (Fig. 5). Thus, our results support a “domain interface model” of phenylalkylamine binding and block, as proposed previously for dihydropyridines (32). The YIFV residues are arranged in two clusters of two amino acids each in IIIS6, but these four residues do not align in precisely the same position in consecutive turns of the \(\alpha\) helix as the YAI motif in IVS6 does (14). Nevertheless, the deviation from strict cylindrical shape of many bundled \(\alpha\) helices in proteins of known structure would allow all four of these residues to contribute to a binding site for phenylalkylamines in the pore of the channel. We propose that the YIFV motif in IIIS6 and the YAI motif in IVS6 act together to form a hydrophobic pocket that stabilizes (-)-D888 bound in the pore and enhances the electrostatic interactions between the pore Glu residues Glu\(^{1118}\) and Glu\(^{1419}\) and the tertiary amino group of (-)-D888 (Fig. 5A). It will be of interest to determine how other structurally related phenylalkylamines of differing affinity interact with these components of the high affinity phenylalkylamine receptor site.

Dihydropyridine and (-)-D888 Binding Sites Are Overlapping But Not Identical—As we have shown in the preceding paper (33), mutation of specific amino acid residues in transmembrane segments IIIS6 and IVS6 greatly reduce the affinity of the \(\alpha\)-type Ca\(^{2+}\) channel for the dihydropyridine PN200-110 as measured by equilibrium binding. The results of mutations in IIIS6 and IVS6 on block by (-)-D888 and binding of PN200-110 are summarized in Fig. 5A. It is clear that the binding sites for these structurally distinct molecules are intricately interwoven, because single mutations at three residues in adjacent positions in IIIS6 and IVS6 (Tyr\(^{1152}\), Ile\(^{1153}\), and Tyr\(^{1465}\)) disrupted both DHP\(^2\) and (-)-D888 binding. However, mutations at other positions had drug-specific effects. For example, mutation of Phe\(^{1164}\), Val\(^{1165}\), Ala\(^{1467}\), and Ile\(^{1470}\) affected block by (-)-D888 but did not significantly affect PN200-110 binding. Conversely, mutations of five amino acids in the central region of IIIS6 (Ile\(^{1156}\), Phe\(^{1158}\), Phe\(^{1159}\), Met\(^{1160}\), and Met\(^{1161}\)) as well as four amino acids in IVS6 (Phe\(^{1462}\), Met\(^{1464}\), Ile\(^{1471}\), and Aan\(^{1472}\)) each had significant effects on DHP binding but not on (-)-D888 block. We therefore suggest that the phenylalkylamines and DHPs bind to different faces of the IIIS6 and IVS6 transmembrane segments and in some cases bind to opposite sides of the same amino acid residues. In this model, the allosteric interactions between bound phenylalkylamines and DHPs would take place over very short distances, possibly separated by no more than the plane of a phenyl ring or the width of an aliphatic side chain.

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