Opposite Effects of a Single IIIS5 Mutation on Phenylalkylamine and Dihydropyridine Interaction with L-type Ca\(^{2+}\) Channels

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Replacement of L-type Ca\(^{2+}\) channel α1 subunit residue Thr-1066 in segment IIIS5 by a tyrosine residue conserved in the corresponding positions of non-L-type Ca\(^{2+}\) channels eliminates high dihydropyridine sensitivity through a steric mechanism. To determine the effects of this mutation on phenylalkylamine interaction, we exploited the availability of Ca\(_{1.2}\)DHP\(^{-/+/−}\) mice containing the T1066Y mutation. In contrast to dihydropyridines, increased protein-dependent binding of the phenylalkylamine (−)[\(^3\)H]devapamil occurred to Ca\(_{1.2}\)DHP\(^{-/−}\) mouse brain microsomes. This effect could be attributed to an at least 2-fold increase in affinity as determined by saturation analysis and binding inhibition experiments. The latter also revealed a higher affinity for (−)-verapamil but not for (−)-gallopamil. The mutation caused a pronounced slowing of (−)[\(^3\)H]devapamil dissociation, indicating a stabilization of the drug-channel complex. The increased affinity of mutant channels was also evident in functional studies after heterologous expression of wild type and T1066Y channels in Xenopus laevis oocytes. 100 μM (−)-verapamil inhibited a significantly larger fraction of Ba\(^{2+}\) inward current through mutant than through WT channels. Our results provide evidence that phenylalkylamines also interact with the IIIS5 helix and that the geometry of the IIIS5 helix affects the access and/or binding of different chemical classes of Ca\(^{2+}\) channel blockers to their overlapping binding domains. Mutation of Thr-1066 to a non-L-type tyrosine residue can be exploited to differentially affect phenylalkylamine and dihydropyridine binding to L-type Ca\(^{2+}\) channels.

Voltage-gated L-type Ca\(^{2+}\) channels (LTCCs)\(^1\) are characterized by their high sensitivity toward Ca\(^{2+}\) channel blockers (CCBs; Ca\(^{2+}\) antagonists). High CCB sensitivity is encoded in their pore-forming α1 subunits (Ca\(_{1.1}\)–Ca\(_{1.4}\) α1), which contain a number of unique amino acid residues critical for the formation of a binding pocket for different chemical classes of CCBs. Clinically relevant are 1,4-dihydropyridines (DHPs; e.g. isradipine and nifedipine), phenylalkylamines (PAAs; e.g. verapamil and gallopamil) and benzothiazepines (BTZs; e.g. (+)-cis-diltiazem). These chemical classes affect each other's binding to the α1-subunit through noncompetitive interactions (1). A detailed molecular analysis of the distinct drug binding domains was performed, which revealed that DHP, PAA, and BTZ binding residues are located within the same regions on α1 subunits and that some residues even participate in the binding of more than one class of CCBs (1–3). This suggested that all three binding domains are located in close proximity to each other and even overlap (Fig. 1). Studies with fluorescently labeled DHPs and BTZs supported this model and provided experimental evidence for a steric interference of bound DHPs with the access pathway for BTZs (4). A multisubsite domain binding model was therefore proposed in which the apparent noncompetitive interactions observed between different classes of CCBs mainly result from steric interactions rather than drug-induced conformational changes (1). This multisubsite binding domain is located in the pore-forming regions of repeats III and IV of the α1-subunit. It allows drug binding to a domain interface, thus facilitating stabilization of closed channel conformations ("domain interface model") (2).

Detailed molecular analysis of amino acid residues involved in drug binding is available for all three chemical classes of CCBs. Altered sensitivity of currents through mutated channels was tested by electrophysiological means after expression of the channel complex in heterologous systems. However, this analysis is complicated by the fact that the apparent sensitivity of CCBs is critically affected by channel gating in an either voltage- or use-dependent manner (5, 6). Mutation-induced changes in channel gating can therefore lead to pronounced alterations in drug sensitivity without necessarily changing binding affinity (6). Radioreceptor assays with recombinant channels directly allow us to measure changes in binding affinity (i.e. the dissociation constant, \(K_D\)) and are therefore a valuable addition to confirm electrophysiological findings. In contrast to the successful application of this approach for DHPs, PAA radioligand binding to recombinant LTCCs has not yet been accomplished. This is not only due to their lower affinity for LTCCs as compared with DHPs (7) but also to higher nonspecific binding of suitable radioligands (−)[\(^3\)H]devapamil or [\(^3\)H]verapamil) to low affinity binding sites on other proteins (8, 9).

In this paper, we exploited a new mouse model in which Thr-1066 in transmembrane segment IIIS5 was mutated to a tyrosine residue (T1066Y) in Ca\(_{1.2}\) α1, the major LTCC isoform expressed in heart and brain (10). The mutation serves as a molecular switch that eliminates high DHP sensitivity by steric hindrance of DHP binding (11). These mutant mice pro-
vide us with the unique opportunity to directly quantify the consequences of this mutation by radioligand binding in native channels of mouse brain membranes. Here we report that Tyr-1066 unexpectedly increased PAA affinity by stabilization of the PAA-channel complex. This clearly shows that PAA can also interact with amino acids in repeat III in 5 and provides an unequivocal example for a residue that is able to control both DHP and PAA interaction. Since a Tyr residue is present in the corresponding position of non-LTCC α1 subunits, this residue must confer some of the known sensitivity of these channels for PAA.

EXPERIMENTAL PROCEDURES

Reagents—(-)[1-3H]Devapamil (80 Ci/mmol) was purchased from Amersham Biosciences (Buckinghamshire, UK). Unlabeled PAA enantiomers were kindly provided by Dr. Glassmann (Medical University of Innsbruck) and Dr. Traut (Knoll AG, Ludwigshafen, Germany).

Animals—The generation and breeding of Ca1.2DHP(-) mice was described recently (10) and was approved by the Austrian Bundesministerium für Bildung, Wissenschaft, und Kultur. To minimize use and breeding of genetically modified animals, the number of experiments had to be kept to a minimum.

Membrane Preparation and Immunoblot Analysis—Brain membranes and membranes from tsA201 cells transfected with 4.5 μg of α1, 3.5 μg of α2-β, 2.5 μg of β, and 4.5 μg of PUC carrier DNA were prepared as described (12). Membrane protein concentrations were determined according to Lowry (13), using bovine serum albumin as a standard. Expression of WT and mutant Ca1.2 α1 (α1, C) subunits was quantified in immunoblots employing affinity-purified anti-α1-C terminus antibody as described previously (14). Premature molecular mass standards (high range; Bio-Rad) separated on the same gel were used to calculate the apparent molecular masses of immunoreactive bands.

Radioceptor Assay—Binding experiments with the phenylalkylamine (-)[1-3H]devapamil were performed in 50 mM Tris-HCl buffer, pH 7.4, supplemented with 0.1 mM phenylmethylsulfonylfluoride and 0.2 mM EDTA. Membrane protein and radioligand concentrations as indicated in the figure legends. Nonspecific binding was determined and subtracted from total binding to yield specific binding. Binding inhibition (and stimulation) studies were performed in the absence (control) and presence of increasing concentrations of unlabeled devapamil serially diluted in MeSO. The maximal MeSO concentration in the assay did not exceed 1% (v/v), which does not affect DHP and PAA binding. For dissociation kinetic experiments, dissociation of the preformed drug-channel complex was monitored by the addition of 3 μM unlabeled devapamil at different time intervals before determining the concentration of bound complexes. The concentration of bound ligand was determined by rapid filtration of the assay mixture through GF/C Whatman filters, pretreated with 0.25% (v/v) polyethyleneimine and 0.25 mg/ml bovine serum albumin for 30 min at 22 °C (15). Filters were washed three times with ice-cold filtration buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl), and filter-bound radioactivity was determined by liquid scintillation counting (16). Binding inhibition (and stimulation) data were fitted to the general dose-response equation (17) to obtain IC50 values and slope factors. Biphasic saturation data were subjected to Scatchard transformation. Mutant-induced changes of a high affinity binding site were estimated from the initial slopes of the Scatchard analysis by linear regression analysis of the five or six data points with the lowest free radioligand concentration (see “Results” and corresponding figure legends). Dissociation rate constants (kdiss) were determined by nonlinear fitting of the data to equations describing mono- or biexponential dissociation reactions as appropriate. Binding parameters were calculated using GraphPad Prism 4.09.

In Situ Hybridization Experiments—In situ hybridization experiments were carried out as previously described (18). [α-32P]dATP end-labeled 45-mer antisense oligonucleotides were synthesized using a terminal transferase kit (Roche Applied Science). The following antisense primers against the mouse Ca1.2 α1 sequence (GenBank accession number L01776; positions 1242–1286 and 1361–1405) were used: 5′-GGG AGC AAG TCC TGC AGC TGC ATT GGC ATT CAT GTT GCC ATG AGC-3′ and 5′-TGT GTG GAA CTG AGC GTA GAG ATG GTT GTG TGG CCA GTA GGC CCC-3′. Construction of α1 cDNAs—Nucleotide and amino acid numbering is according to sequence accession no. X15539. An Acc65I (‘5’ polynucleotide)-XbaI (‘3’ polynucleotide) poly(A) sequence fragment (210 bp) was coligated with a Sall-Acc65I (‘5’ polynucleotide-‘3’ polynucleotide) fragment of the rabbit cardiac Ca1.2 α1 subunit cDNA (19) inserted into the mammalian expression vector pGFP (a gift from M. Grabner, Medical University of Innsbruck), and the XbaI-Xbal (‘5’-‘3’ polynucleotide) cut mammalian expression vector pCNeo (Invitrogen), yielding Ca1.2pApCNeo. Mutation T1066Y was initially introduced into Ca1.2pApGFP by overlap extension PCR using an AIII-Th1111I cassette (nucleotides 2880–3579), yielding Ca1.2TPygFP. Subsequently, Ca1.2TPygCNeo was constructed for heterologous expression in Xenopus laevis oocytes by transfecting an EcoRI-SacI (nucleotides 2405–4037) fragment from Ca1.2TPygFP to Ca1.2pApCNeo.

Electrophysiological Measurements in X. laevis Oocytes—Capped run-off poly(A+) cRNA transcripts from XbaI-linearized cDNA templates of WT and the T1066Y mutant were synthesized according to Krieg and Melton (20). α1 cRNA was co-injected with β1, β2, and αβ2 subunit CRNAs into stage V-VI oocytes from X. laevis. To avoid bias, the experimenter was blinded to the injected oocytes until data analysis was finished.

2–3 days after cRNA injection, inward barium currents (Iba) through voltage-gated Ca2+ channels were measured at room temperature using the two-microelectrode voltage clamp technique as described previously (11). To quantify endogenous Iba, X. laevis oocytes injected only with β1, αβ2, and αβ2 were analyzed in parallel. Only oocytes expressing peak Iba through recombinant Ca2+ channels at least 7 times larger than the highest endogenous currents were included in the analysis. Data analysis and acquisition was performed using the pClamp software package (version 6.0; Axon Instruments). Leakage correction was performed by adjusting the current traces by a factor calculated from the difference between the leak at ~80 mV and ~90 mV, respectively. The extracellular solution contained 40 mM Ba(OH)2, 50 mM NaOH, 2 mM CsOH, and 5 mM Hepes (pH adjusted to 7.4 with methanesulfonic acid). The voltage recording and current-injecting microelectrodes were filled with 2.8 mM CsCl, 0.2 mM CsOH, 10 mM Hepes, and 10 mM EGTA (adjusted to pH 7.4 with HCl) and had resistances of 0.7–5 MΩ.

Before starting an experiment, the oocytes were held at ~80 mV until Iba stabilized. Use-dependent modulation of peak Iba by 100 μM (-)-verapamil was measured during 20 consecutive 100-ms pulses applied at a frequency of 0.66 Hz from a holding potential of ~80 mV to a test pulse corresponding to the peak potential of the current-voltage relations. Control traces were recorded twice in the absence of drug separated by a 5-min interval to allow for complete channel recovery. The same protocol was then repeated after perfusion of the oocyte with drug (gray), and both DHP and PAA (open) binding affinity (1, 2). Only repeats III and IV are illustrated.

![Drug binding residues in LTCC α1 subunits. Shown is a conventional putative folding structure of LTCC α1 subunits, with circles indicating the position of residues crucial for DHP (black), PAA (gray), and both DHP and PAA (open) binding affinity (1, 2).](image)
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RESULTS

Fig. 1 illustrates the location of residue Thr-1066 (numbering according to Ca\(_{1.2}\) (10) in the putative folding structure of LTCC \(\alpha_1\)-subunits. Mutation to tyrosine eliminates high affinity for DHPs as assessed by radioligand binding and functional studies (3, 10).

To test whether III S5 mutation T1066Y affects PAA interaction with LTCCs, we measured specific (\(-\))-[\(^{3}H\)]devapamil binding to brain microsomes prepared from WT mice, Ca\(_{1.2}\) DHP\(^{+/-}\) mice heterozygous for the mutation (\(+/-\)), and homozygous Ca\(_{1.2}\) DHP\(^{+/-}\) mice (\(-/-\); Fig. 2). Binding was linearly dependent on membrane protein concentrations (Fig. 2A). In contrast to DHPs (10), protein-dependent specific binding was significantly higher for heterozygous and mutant mouse brain membranes than for WT at equal radioligand concentrations (Fig. 2, A and B).

The observation of a higher specific (\(-\))-[\(^{3}H\)]devapamil binding signal for Ca\(_{1.2}\) DHP\(^{+/-}\) and Ca\(_{1.2}\) DHP\(^{-/-}\) mice raised the question of whether this effect was due to an increase in binding affinity or resulted from a mutation-induced up-regulation of Ca\(_{1.2}\) channels. We considered the latter possibility unlikely, because we found no evidence of increased immunoreactivity for mutated Ca\(_{1.2}\) \(\alpha_1\) in brain and heart membranes of Ca\(_{1.2}\) DHP\(^{-/-}\) mice in an earlier study (10); nor did we detect increased expression of recombinant mutant mouse Ca\(_{1.2}\) DHP\(^{-/-}\) channel complexes after heterologous expression in tsA201 cells (Fig. 3A). Furthermore, the mutation did not alter the neuronal expression pattern of Ca\(_{1.2}\) mRNAs as determined by \(in situ\) hybridization experiments in whole brain sections (Fig. 3B). Thus, it appeared more likely that the mutation enhanced binding by increasing (\(-\)) devapamil binding affinity. Fig. 4 illustrates the inhibition of specific (\(-\))-[\(^{3}H\)]devapamil binding to WT and mutant brain LTCCs by the more potent (\(-\))-enantiomers of devapamil, verapamil, and gallopamil. These PAA s only differ with respect to the number of methoxy substituents at their phenyl rings (7). For (\(-\))- devapamil and (\(-\))-verapamil a reproducible decrease of the IC\(_{50}\) values was observed in three independent experiments (see the legend to Fig. 4). This increase in affinity was also observed in the presence of a physiologically relevant (1 mM) concentration of CaCl\(_2\) (see the legend to Fig. 4), which affects PAA interaction with the extracellularly oriented selectivity filter glutamate (2). In contrast, the IC\(_{50}\) was not increased for (\(-\))-gallopamil (Fig. 4). These data confirmed the higher binding signal observed for (\(-\))-[\(^{3}H\)]devapamil and suggested that mutation T1066Y increases the affinity of LTCCs for (\(-\))-devapamil and (\(-\))-verapamil.

To further substantiate this finding, we attempted to determine the KD for (\(-\))-[\(^{3}H\)]devapamil in saturation experiments, although the low slope factor in displacement studies (Fig. 4A) suggested that it labeled more than one population of sites in mouse brain membranes. As shown in Fig. 5, this was indeed the case. Such low affinity sites for (\(-\))-[\(^{3}H\)]devapamil have previously been reported in mammalian brain (23) and in car-

Fig. 2. Equilibrium binding of (\(-\))-[\(^{3}H\)]devapamil to mouse brain membranes. A, 0.65 nm (\(-\))-[\(^{3}H\)]devapamil were incubated with increasing concentrations of brain membrane protein from Ca\(_{1.2}\) \(\alpha_1\) WT, heterozygous (+/-), and homozygous Ca\(_{1.2}\) DHP\(^{+/-}\) mice (-/-) for 60 min at 22 °C in a final assay volume of 0.5 ml. Nonspecific binding was measured in the presence of 3 \(\mu\)M unlabeled (\(-\))-devapamil. The following relative slope values were calculated by linear curve fitting, given in percentage of wild type: WT, 100%; +/-, 151%; -/-, 205%. One of five similar experiments is shown. Nonspecific binding was 519–694 dpm at the highest membrane protein concentration. B, (\(-\))-[\(^{3}H\)]devapamil (0.59–0.65 nm) binding to wild type, heterozygous, and mutant mouse brain membranes at equal protein concentrations (0.01–0.32 mg/ml): WT, 100%; +/-, 162.7 ± 13.5%; -/-, 250.2 ± 48.2%, n = 5 (means ± S.E.). *, significant difference from WT (p < 0.05).

Fig. 3. Effects of mutation T1066Y on Ca\(_{1.2}\) subunit expression. A, heterologous expression of recombinant T1066Y and WT Ca\(_{1.2}\) \(\alpha_1\) subunit protein in tsA201 cells. Cells were transfected with Ca\(_{1.2}\) \(\alpha_1\) (WT), T1066Y \(\alpha_1\), or the expression vector pGFP (mock) together with \(\beta_1\), \(\beta_2\), and \(\beta_3\) subunit cDNA. Mutant and WT Ca\(_{1.2}\) \(\alpha_1\) subunit expression was analyzed in immunoblots using 10 \(\mu\)g of membrane protein prepared from lysed cells after separation on 7% SDS-polyacrylamide gels and detected by the sequence-directed antibody anti-Ca\(_{1.2}\) (10) in the putative folding structure of Ca\(_{1.2}\) (10).

The migration of specific Ca\(_{1.2}\) \(\alpha_1\) immunoreactivity (arrow) and of prestained marker proteins is indicated. One of two independent transfection experiments yielding similar results is shown. B, expression of Ca\(_{1.2}\) \(\alpha_1\) subunit mRNAs in brains of WT and Ca\(_{1.2}\) DHP\(^{-/-}\) mice. \(in situ\) hybridization was carried out as described under “Experimental Procedures.” One of three experiments yielding similar results is shown.
membranes as compared with WT (Fig. 6). After 20 min, almost complete dissociation of radioligand occurred from WT channel complexes, whereas more than 50% of specific binding to the mutant channel remained (Fig. 6A). Whereas monophasic dissociation of (-)-[3H]devapamil from mutant channels occurred with a dissociation rate constant ($k_{-1}$) of 0.0209 ± 0.0023 min$^{-1}$ ($n = 5$), $k_{-1}$ was 5–10-fold higher for WT (see legend to Fig. 6B). This suggests that the mutation stabilizes (-)-[3H]devapamil binding to LTCCs in accordance with the observed decrease in $K_D$.

The interesting finding that a single mutation can increase the affinity for PAAs but eliminates high affinity for DHPs in radioligand binding experiments (10) enabled us to reversibly label WT and mutant LTCCs with (-)-[3H]devapamil and monitor the loss of DHP binding affinity for the T1066Y channel over a broad concentration range. This was possible by exploiting the known noncompetitive inhibition of (-)-[3H]devapamil binding to L-type channels by DHPs. Isradipine partially inhibited (-)-[3H]devapamil binding to WT channels (Fig. 7) in a highly stereoselective manner ($\pm$-isradipine: $IC_{50} = 0.16 \pm 0.02$, slope factor = 1.62 ± 0.30; percentage inhibition = 49.1 ± 4.2, $n = 5$; (-)-isradipine: $IC_{50} = 8.78 \pm 2.12$, slope factor = 1.06 ± 0.18; percentage inhibition = 37.4 ± 10.3; $n = 3$). No inhibition was found for mutant channels up to 10 $\mu M$ concentrations (Fig. 7). Thus, mutation T1066Y dramatically reduces DHP antagonist sensitivity, completely preventing noncompetitive inhibition of PAA binding by both isradipine enantiomers even at micromolar concentrations.

To prove the increased PAA affinity also in functional studies, we expressed WT and mutant Ca$_{1.2}$DHP$^-\tau$ subunits in X. laevis oocytes together with $\beta_2$ and $\alpha_\delta$ subunits and measured the inhibitory effect of 100 $\mu M$ (-)-verapamil on channel currents using Ba$^{2+}$ as a charge carrier. Verapamil was used, because our binding experiments indicated a slightly higher increase in binding affinity of the (-)-enantiomer for the mutant channel (Fig. 4), and only small amounts of (-)-devapamil were available for our studies. PAAs are use-dependent blockers of LTCCs. PAA sensitivity depends on depolarization frequency and pulse duration (24), making it difficult to assess small differences in affinity by generating concentration-response curves. We therefore selected a pulse protocol in which $I_{Na}$ decay during pulse trains was minimal (<7%; see legend to Fig. 8), and thus even a small increase in use-dependent block by (-)-verapamil should be de-
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**Figure 6. Dissociation kinetics of (−)\[^{3}H\]devapamil binding to mutant and WT brain membranes.** 0.05–0.07 mg/ml membrane protein from WT and mutant (−/−) mouse brain preparations were preincubated with 0.59–0.61 nM (−)\[^{3}H\]devapamil until equilibrium was reached. Radioligand dissociation was initiated by rapid addition of a final concentration of 3 μM unlabeled racemic devapamil at the indicated time periods before filtration. A, specific binding (expressed as percentage of equilibrium binding \(B_0\)) after the indicated time periods. Pooled data from five independent experiments are shown; **, \(p < 0.01\). B, time course of (−)\[^{3}H\]devapamil dissociation. Monophasic fits of the normalized dissociation data yielded the following \(k_{-1}\) values: for WT (○), \(k_{-1}\) = 0.273 min\(^{-1}\); for −/− (□), \(k_{-1}\) = 0.019 min\(^{-1}\). In a second experiment, which allowed resolution of the fast dissociating component in WT membranes, a \(k_{-1}\) value of 0.107 min\(^{-1}\) was calculated. Monoeponential fits did not account for 4–10% of the binding signal, indicating the possibility of slower dissociation from a small fraction of binding sites. Since this would not affect the interpretation of our data, this finding was not further investigated. The mean \(k_{-1}\) for mutant channels was 0.0209 ± 0.0023 min\(^{-1}\) (n = 5). For the illustrated experiment, total and nonspecific binding under equilibrium conditions were as follows (in dpm/0.5 ml): for WT, total = 3486, nonspecific = 1007; for mutant, total = 5888, nonspecific = 1119.

**Figure 7. Stereoselective modulation of (−)\[^{3}H\]devapamil binding to mutant and WT mouse brain membranes by isradipine.** Radioligand and membrane concentrations were 0.63–0.68 nm and 0.18 mg/ml, respectively. One of three (−)-isradipine or five (±)-isradipine) experiments is shown (for statistics, see "Results"). The following IC\(_{50}\) values were calculated by nonlinear curve fitting: for WT, (±)-isradipine (▲), IC\(_{50}\) = 0.11 nm (slope = 0.86), 56.8% inhibition of control binding; for (−)-isradipine (●), IC\(_{50}\) = 4.54 nm (slope = 0.83), 57.9% inhibition of control binding. For mutant channels, no evidence was found for binding inhibition by (±)-isradipine (▲) (percentage of control binding: 10 nm, 99.7 ± 0.85, n = 4; 1 μM, 97.2–100.7, n = 2) or (−)-isradipine (●) (percentage of control binding: 1 μM, 97.2 ± 3.6, n = 3).

tectable. This was achieved by applying trains of 20 consecutive 100-ms pulses at a frequency of 0.66 Hz from a holding potential of −80 mV to a test pulse corresponding to the peak potential of the current-voltage relations. Under these conditions, 100 μM verapamil inhibited 23.7 ± 2.56% (n = 5) of \(I_{\text{Ba}}\) in WT channels (Fig. 8). A significantly (p < 0.01) larger inhibition (42.94 ± 3.70%; n = 7) was induced in mutant channels. Although the complexity of the use-dependent verapamil inhibition does not allow us to quantitate the apparent sensitivity difference, these functional data nicely confirm our results obtained by radioligand binding studies.

**Discussion**

By demonstrating that a tyrosine residue in position 1066 increases PAA affinity, we provide novel insight into the organization of the LTCC multisite drug binding domain. So far, no interactions of PAAs with segment IIIS5 have been demonstrated. We show that the bound PAA molecule is well within reach of IIIS5 amino acid side chains and that a tyrosine side chain (as present in non-L-type channel α₁-subunits) is able to interact both with PAAs and DHPs exerting opposite effects on binding affinity.

Our data also provide some indirect evidence concerning the orientation of the Tyr-1066 (numbering according to Cav1.2) side chain with respect to the drug binding pocket. One possibility is that it sterically blocks DHP binding by protruding deep into the multisubsite domain. In this case, we would also expect a steric interference with PAA binding based on the facts that (i) the PAAs examined here are larger than DHPs (such as isradipine) and (ii) the DHP and PAA binding domains must considerably overlap (1, 2). Our data therefore suggest a model (Fig. 9) in which the tyrosine side chain is oriented more toward the access pathway of DHPs and/or those regions of the multisubsite domain that are preferentially occupied by the DHP molecule. Opposite effects of this residue on DHP and PAA binding are also in agreement with previous electrophysiological studies, which used permanently charged drugs to demonstrate that DHPs and PAAs approach their binding domains from opposite sides of the channel. Whereas DHPs rely on an extracellular access pathway (25, 26), PAAs block the channel through a cytoplasmic approach (24, 27). In the proposed model, such separate access pathways would allow the (also more flexible) PAA molecule to still reach its site inside the mutant channel and simultaneously provide a close interaction with the Tyr-1066 side chain, thereby stabilizing binding and decreasing \(K_D\). A stabilization of the PAA-channel complex was evident from our kinetic studies, which revealed a pronounced slowing of (−)\[^{3}H\]devapamil dissociation from mutant brain membranes. The mutation may not only slow dissociation, but it seems to also slow association of (−)-devapamil, because the slowing of dissociation (5–10-fold) appeared larger than the increase in binding affinity.

Because we do not yet know how the S5 and pore-forming S6 helices are packed against each other, it is possible that the IIIS5 mutation affects preferentially the geometry of the binding site for DHPs. However, due to the strong overlap of the two binding domains, we consider this rather unlikely. S6 helices also control the gating and pore properties of the channel, and thus conformational changes may also alter the biophysical channel properties. However, we and others (see Ref. 10 and references cited therein) did not find evidence for gating changes of the mutant channel.

Note that our detailed analysis of the PAA interaction of
mutation T1066Y was only possible because it was introduced in mice in vivo and accessible to established (-)-[3H]devapamil binding assays in wild type and mutant mouse brain membranes. So far, no binding assay has been established to probe PAA binding to recombinant LTCCs expressed in heterologous systems as described for DHPs (5). This is mainly due to a higher nonspecific binding and the 4–10-fold lower binding affinity of (-)-[3H]devapamil (K_D = 1–2.5 nM) as compared with DHPs ((+)-[3H]isradipine; K_D = 0.25–0.5 nM) (7, 28).

Our data also provide a molecular basis for the finding that N- and P/Q-type Ca^{2+} channels exhibit some affinity for PAAAs, such as devapamil or verapamil (29–31), but not for DHPs. It is known that some drug binding residues of the PAA binding domain are conserved between LTCC and non-LTCC (e.g. Cav2.1 and Cav2.2) α_{1} subunits. Examples are Tyr-1151, Phe-1172, and Val-1173 in IIIS6. Like our mutant channels, non-LTCCs contain a tyrosine residue in the position corresponding to L-type Thr-1066. Our data provide evidence that this tyrosine together with the other conserved PAA binding residues mediates the weak PAA sensitivity of non-LTCCs.

Two-dimensional models of LTCC drug binding domains exist (12, 32) but are not yet suitable for rational drug design because they are based on the crystal structure of structurally related bacterial K^{+}-channels. Based on the rapid progress...

FIG. 8. Use-dependent block of WT Ca_{1.2} and Ca_{1.2} containing the T1066Y mutation by (-)-verapamil after expression in X. laevis oocytes. WT (Ca_{1.2WT}) and mutant (Ca_{1.2Thr1066Tyr}) α_{1} subunits were expressed with auxiliary subunits in X. laevis oocytes as described under “Experimental Procedures.” Use-dependent modulation of peak I_{Ba} by (-)-verapamil was measured during 20 consecutive 100-ms pulses applied at a frequency of 0.66 Hz from a holding potential of −80 mV to a test pulse corresponding to the peak potential of the current-voltage relations before and 5 min after perfusion with 100 μM (-)-verapamil. A, use-dependent block was expressed as the percentage decrease of peak I_{Ba} during the last test pulse of the train compared with I_{Ba} during the first train. **, p < 0.01. B, representative current traces are shown. I_{Ba} values during the first (1) and last (20) pulse of the train are indicated.

FIG. 9. Model demonstrating the DHP access pathway blockade by the Tyr-1066 side chain. Whereas a threonine residue in position 1066 allows access and binding of both DHPs and PAAs from the extracellular (25, 26) and intracellular side (24, 27), respectively (left and middle), DHP binding and/or access is inhibited by the phenyl ring of tyrosine in position 1066 (right). At the same time, the tyrosine side chain increases PAA binding affinity and stability (right).

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\[2\] I. Huber and J. Striessnig, unpublished observations.
made in elucidating the three-dimensional structure of large ion channels, it is likely that a higher resolution structure will soon also become available for voltage-gated Ca^{2+} channels (33, 34). In combination with detailed maps of the interaction sites of LTCC modulators with individual amino acids, this will not only allow the elucidation of the exact molecular mechanism for LTCC block by these drugs but eventually may also facilitate the rational development of selective blockers of other Ca^{2+} channel \alpha_1 subunits or perhaps of other structurally related members of the cation channel family.

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