Nine L-type Amino Acid Residues Confer Full 1,4-Dihydropyridine Sensitivity to the Neuronal Calcium Channel $\alpha_{1A}$ Subunit

ROLE OF L-TYPE MET$^{1188e}$

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Pharmacological modulation by 1,4-dihydropyridines is a central feature of L-type calcium channels. Recently, eight L-type amino acid residues in transmembrane segments III$S_5$, III$S_6$, and IV$S_6$ of the calcium channel $\alpha_{1A}$ subunit were identified to substantially contribute to 1,4-dihydropyridine sensitivity. To determine whether these eight L-type residues (Thr$^{1066}$, Gln$^{1070}$, Ile$^{1180}$, Tyr$^{1183}$, Met$^{1491}$, Ile$^{1497}$, and Ile$^{1498}$; $\alpha_{1C,a}$ numbering) are sufficient to form a high affinity 1,4-dihydropyridine binding site in a non-L-type calcium channel, we transferred them to the 1,4-dihydropyridine-insensitive $\alpha_{1A}$ subunit using site-directed mutagenesis. 1,4-Dihydropyridine agonist and antagonist modulation of barium inward currents mediated by the mutant $\alpha_{1A}$ subunits, coexpressed with $\alpha_{a\delta}$ and $\beta_{1A}$ subunits in Xenopus laevis oocytes, was investigated with the two-microelectrode voltage clamp technique. The resulting mutant $\alpha_{1A,\text{DHPi}}$ displayed low sensitivity for 1,4-dihydropyridines. Analysis of the 1,4-dihydropyridine binding region of an ancestral L-type $\alpha_{1C,a}$ subunit previously cloned from Musca domestica body wall muscle led to the identification of Met$^{1188}$ ($\alpha_{1C,a}$ numbering) as an additional critical constituent of the L-type 1,4-dihydropyridine binding domain. The introduction of this residue into $\alpha_{1A,\text{DHPi}}$ restored full sensitivity for 1,4-dihydropyridines. It also transferred functional properties considered hallmarks of 1,4-dihydropyridine agonist and antagonist effects (i.e. stereoselectivity, voltage dependence of drug modulation, and agonist-induced shift in the voltage-dependence of activation). Our gain-of-function mutants provide an excellent model for future studies of the structure-activity relationship of 1,4-dihydropyridines to obtain critical structural information for the development of drugs for neuronal, non-L-type calcium channels.

Voltage-dependent calcium channels are activated by membrane depolarization and mediate the rapid and selective entry of calcium into excitable cells. The subsequent rise in intracellular calcium triggers a variety of cellular responses, including excitation-contraction coupling, excitation-secretion coupling, synaptic plasticity, and the modulation of transcription events (for a review, see Refs. 1 and 2). On the basis of electrophysiological and pharmacological criteria, voltage-dependent calcium channels are classified into L-, N-, T-, P/Q-, and R-type channels (reviewed in Refs. 3 and 4). The heterologomeric channel complexes are composed of a pore-forming $\alpha_{1}$ subunit in combination with accessory subunits ($\alpha_{a\delta}$, $\beta$, and in skeletal muscle $\gamma$), which modulate the pharmacological and kinetic channel properties (1, 4). Molecular cloning and heterologous expression experiments have revealed that three classes of $\alpha_{1}$ subunits ($\alpha_{1C,5}$ in heart, smooth muscle, and neurons; $\alpha_{1S,6}$ in skeletal muscle; and $\alpha_{1P,7}$ in neuroendocrine cells) form L-type calcium channels (3). They are distinguished from the other types by their high sensitivity to 1,4-dihydropyridines, phenylalkylamines, and benzothiazepines (8), which are used therapeutically for the treatment of a variety of cardiovascular disorders (9). 1,4-Dihydropyridine (DHP) antagonists stabilize the inactivated state of the channel (10), whereas DHP agonists promote the open state (11). However, the molecular mechanism of channel modulation mediated by these drugs has yet to be completely elucidated.

The DHP binding domain is located on the $\alpha_{1}$ subunit (12). It is tightly coupled to a high affinity calcium binding site (13) representing the ion selectivity filter (14–16). One essential requirement to fully understand the molecular mechanism of channel modulation is the identification of amino acid residues that mediate DHP agonist and antagonist effects. Photoaffinity labeling, combined with antibody mapping (12) as well as construction of chimeric $\alpha_{1}$ subunits (17) identified parts of the high affinity DHP binding domain. Using a gain-of-function approach, we have shown that introducing only as little as 9.4% L-type sequence (including transmembrane segments III$S_5$, III$S_6$, and IV$S_6$) is sufficient to transfer DHP sensitivity to the DHP-insensitive class A (BI-2) calcium channel $\alpha_{11}$ subunit (18). Subsequently, we demonstrated that two amino acid residues of segment III$S_5$ are critical for the DHP interaction (19). In transmembrane segments III$S_6$ and IV$S_6$ of the $\alpha_{11}$ subunit, six other L-type amino acid residues were identified to be required for DHP binding by creating chimeras and mutants that were monitored for a reduction or loss of DHP sensitivity (20, 21).

Here we used a gain-of-function approach to investigate whether these eight L-type residues, when removed from their natural sequence environment, are able to form a functional...
DHP interaction domain. The DHP-insensitive α IA subunit was chosen as the acceptor molecule (22, 23, 18). We discovered that an additional methionine residue in transmembrane segment III/S6 of the L-type calcium channel is also required for DHP sensitivity. Together, these nine L-type amino acid residues of the resulting motif allow for the construction of a fully functional agonist- and antagonist-sensitive DHP binding pocket in α IA subunits.

EXPERIMENTAL PROCEDURES

Construction of Chimeric and Mutant α IA cDNAs—Class AL-type chimeric α subunits derived from the DHP-insensitive rabbit brain class A calcium channel (BI-2) α IA (A; Ref. 22) and the Musca domestica (housefly) muscle ancestral L-type calcium channel α IM (M; Ref. 24) as well as mutants thereof were constructed by using the “gene SOEing” technique (25). “Silent” restriction endonuclease cleavage sites generated by polymerase chain reaction in previous cloning steps (18) are indicated by asterisks. Amino acid and nucleotide numbering is given in parenthesis.

For AL12m, amino acid composition was as follows: A (amino acids 1–1388), M (amino acids 922–1056), A (amino acids 1521–1723), M (amino acids 1821–2424). Nucleotide 3543A was digested with the EcoRV-Cla I fragment (nucleotides 2956M–4925A), generated by “gene SOEing” (overlap: amino acids 3357M/4857A) into the NheI (nucleotide 3543A) and Cla I (nucleotide 4925A) sites of AL9 (18). The following steps were performed with the resulting intermediate clone: the ligation product from the Cla I-Nsi I fragment of A (nucleotides 4925–5456) plus the Pst I-Spe I fragment of M (nucleotides 4011–4307) was digested with the Xba I-Sph I fragment of A (nucleotides 5773–5862) into the Cla I (nucleotide 4925A) and Sph I (nucleotide 5862A) sites of the intermediate clone (note the compatibility of the following restriction endonuclease sites: Hpa I and EcoRV, Nsi I and Pst I; Spe I and Xba I). For AL12m/V1048M, the single point mutation was introduced by using a Hpa I-Kpn I cassette (nucleotides 3714M and 4023M, respectively) in AL12m.

For AL12m/V1352M and AL12m/S1355A, the single point mutation was introduced by using a Kpn I-Bgl II cassette (nucleotides 4023M and 6168A, respectively) cassette in AL12m.

For α IA-DHP, the following amino acid numbering is according to α IA sequence (22). Single point mutations F1504I and F1507I were introduced into segment IIS6 by using a Sal I-Cla I cassette of A (nucleotides 2449–4295) in AL15 (18), yielding chimera AL15 (18) in Single point mutations I1804Y, F1805M, M1811I, and L1812I were introduced into using a Hpa I-Kpn I cassette (nucleotides 3714M and 4023M, respectively) cassette in AL12m.

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RESULTS

Transfer of Eight L-type Amino Acids Creates a Mutant α IA Subunit with Weak Sensitivity to the DHPs (BayK 8644 and Irsadipine)—Using site-directed mutagenesis, we transferred the L-type residues Thr 1066, Gln 1070, Ile 1180, Ile 1183, Tyr 1189, Met 1493, Ile 1497, and Ile 1498 (numbering according to α IA-cDNA) to the α IA subunit by replacing the corresponding non-L-type residues. The resulting mutant α IA-DHP (Fig. 1A) was coexpressed with auxiliary α δ and β IA subunits in Xenopus oocytes to test if these residues can form an L-type DHP binding domain within the background of α IA sequence. α IA-DHP gave rise to barium inward currents with a threshold of activation between −35 and −25 mV (Fig. 1B). The midpoint voltage of current activation was −8.6 ± 1.0 mV (n = 13), and maximal I m amplitudes were reached at test potentials between 0 and 10 mV. I m modulation by the standard DHP probe BayK 8644 (agonist) and (-)-irsadipine (antagonist) was considerably reduced when compared with recombinant L-type calcium channels expressed in Xenopus oocytes (18). Peak I m of α IA-DHP, measured at peak potentials of the current-voltage relations, was blocked by (-)-irsadipine (Fig. 1B, upper panel) with a low apparent potency, yielding an IC 50 of −100 μM (Fig. 4B). The midpoint voltage of peak I m by 10 μM (-)-BayK 8644 was only 1.0 mV (Fig. 1A), while test potentials 10 mV negative to the peak of the current-voltage relations (Fig. 1B, lower panel). Most features of BayK 8644 modulation were not detectable, such as the agonist-induced slowing of macroscopic I m-activation at threshold potentials (not shown) as well as a slowing of channel deactivation (Fig. 1B, lower panel). 10 μM (-)-BayK 8644 failed to significantly
Transfer of DHP Sensitivity

Chimera AL12m Reveals the Importance of a Methionine Residue in IIIS6 for DHP Sensitivity—Recently, we have cloned the ancestral L-type α1M subunit from housefly (M. domestica) body wall muscle (24). Similar to L-type α1 subunits derived from vertebrate skeletal muscle (6, 31), functional expression of α1M in *Xenopus* oocytes failed (24). We therefore attempted to investigate the DHP sensitivity of α1M by means of a chimeric calcium channel α1 subunit, termed AL12m (Fig. 2A), that was constructed in analogy to the DHP agonist- and antagonist-sensitive chimeras AL12h and AL12s (18). As shown in Fig. 2B, the *I*Ba-modulation of AL12m differed from AL12h and AL12s. *I*Ba of AL12m was clearly blocked by (+)-isradipine, but stimulation by (+)-BayK 8644 could not be detected.

We exploited this absence of agonist sensitivity to identify additional amino acid residues that are involved in the DHP interaction. Sequence alignments of transmembrane segments IIIS6 and IVS6 from cardiac and skeletal muscle L-type α1 subunits with α1M revealed three amino acid divergences (Fig. 2C) in α1M, located within previously described core regions of the DHP interaction domain (20, 21). For the *Musca* channel, two Met to Val exchanges were identified, one each in IIIS6 and IVS6, and an Ala to Ser conversion was also found in IVS6. Interestingly, each of these residues (Met1188, Met1491, and Ala1404, α1C-a numbering) is highly conserved among vertebrate L-type channel α1 subunits and possesses a highly conserved counterpart (Val512, Phe1805, and Ser1808, respectively; α1C-a numbering) in non-L-type α1 subunits. We therefore converted single amino acid residues in AL12m to vertebrate L-type sequence and tested the DHP sensitivity of the resulting chimeras AL12m/V1048M, AL12m/V1352M, and AL12m/S1355A (numbering according to α1M; Ref. 24). Both Val to Met conversions (but not the Ser to Ala substitution) resulted in an increased agonist sensitivity (Fig. 2E). In contrast to AL12m-mediated currents, 10 μM (±)-BayK 8644 stimulated the *I*Ba peak amplitudes of AL12m/V1048M and AL12m/V1352M 1.6 ± 0.1-fold (*n* = 4), and 1.4 ± 0.1-fold (*n* = 4), respectively. Although BayK 8644-mediated agonist modulation of these AL12m mutants did not reach the extent observed for recombinant L-type channels or the chimeras AL12s and AL12h (18), each of the methionine residues played an important role for agonist sensitivity. It remains to be investigated whether simultaneous conversion of both residues could elicit full BayK 8644 sensitivity. The inhibition of peak *I*Ba by 10 μM (+)-isradipine was 70 ± 6% (*n* = 5), 69 ± 7% (*n* = 4), 80 ± 3% (*n* = 3), and 64 ± 6% (*n* = 4) for AL12m, AL12m/V1048M, AL12m/V1352M, and AL12m/S1355A (Fig. 2D), and therefore comparable (*p* < 0.01) for AL12m and the single mutants derived thereof. These findings suggested that these two methionine residues play a prominent role in agonist modulation, at least within a sequence environment that presumably contains additional, low affinity interaction sites for DHP antagonists in the SS-S6 linker of domain IV, similar to the α1C-a-chimeras AL10 and AL13 (18).

The substantial contribution of Met1491 (AL12m/V1352M) in transmembrane segment IVS6 to agonist sensitivity is in agreement with previously published studies (20, 21). In contrast, the importance of Met1188 (numbering according to α1C-a) in IIIS6 for DHP agonist modulation represents an entirely new finding. Of these two L-type residues, only the methionine in IVS6 was present in α1A-DHP. Consequently, the absence of Met1188 could be responsible for the lower DHP sensitivity and an absence of both an agonist-induced shift in activation and slowing of channel deactivation of this mutant. To test this possibility, we introduced Met1188 into α1A-DHP. The resulting mutant was termed α1A-DHP.

Met1188 Is Required for Full Agonist and Antagonist Sensi-
activity in the mutant -Subunit—Fig. 3A shows a schematic drawing of with Met1188 in the IIIS6 segment. Coexpression of with and subunits resulted in barium inward currents similar to . The threshold of current activation was between −35 and −25 mV, and the half-activation potential of −10.3 ± 1.3 mV (n = 12) was indistinguishable (p > 0.01) from the midpoint voltage of current activation determined for . Maximal current amplitudes were also reached at test potentials between 0 and 10 mV (Fig. 3B). Macropscopic were inactivated (expressed as percentage of peak current decay during 100 ms at test potentials corresponding to the peak of the current-voltage relations) was less in (32 ± 1%; n = 12) than in (56 ± 1%; n = 13). Differences in the response of compared with , to the agonist (−)-BayK 8644 and the antagonist (−)-isradipine were obvious. The addition of 10 μM (−)-BayK8644 resulted in a 3.7 ± 0.3-fold (n = 5) stimulation of peak measured at test potentials of 10 mV negative to the peak potential of the current-voltage relations (Fig. 4A). This amount of peak increase is significantly (p < 0.01) higher than that of mediated (Fig. 4A) and corresponds to the extent expected for recombinant L-type channels expressed in (18). Other classical features of BayK 8644 modulation were also restored in . We observed a characteristic slowing in the activation kinetics, determined as a 62 ± 28-ms (n = 3) difference in the time to peak with and without drug at test potentials close to the midpoint voltage of the current activation curve. (−)-BayK 8644 shifted the activation curve by 7.1 ± 1.8 mV (n = 3) in the hyperpolarized direction and induced a moderate but detectable slowing of deactivation (Fig. 3B, lower panel). The stimulatory effect of (−)-BayK 8644 was concentration-dependent (Fig. 4A). A complete concentration-response relationship could not be obtained due to the limited solubility of (−)-BayK8644 in the recording solution. Nevertheless, the data obtained for (Fig. 4A) indicate that Met1188 critically contributes to agonist modulation by (−)-BayK 8644. Despite a 1.6 ± 0.1-fold (n = 7) increase of peak at 10 μM, (−)-BayK 8644 failed to appreciably shift the activation curve and slowed neither the activation kinetics nor the deactivation kinetics of (Fig. 1). Met1188 therefore is likely to at least contribute to the ability of L-type calcium channels to display these features of agonist modulation, but this hypothesis remains to be confirmed by testing for the functional consequences of a respective Met to Val or Met to Ala mutation in or.

To investigate the effect of the Val to Met conversion on DHP antagonist sensitivity, we assessed the concentration-dependent inhibition of (−)-isradipine in comparison with (Fig. 4B). The apparent IC50 values of ~10 μM for (Fig. 4B) and 67 nM for indicate that exhibits also a substantial impact on DHP antagonist sensitivity.

Features of the Modulation of (−)-Isradipine and (−)-BayK 8644—Fig. 5 illustrates the antagonist and agonist effects on that are typical for L-type calcium channel modulation. Stereoselectivity represents a core requirement for

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**Fig. 2. Chimera AL12m uncovers the importance of Met1188 for DHP sensitivity.** A, schematic representation of the chimeric calcium channel subunit AL12m, which was constructed by introducing sequence stretches (black segments and bold lines) from the ancestral L-type subunit ( ) (Ref. 24) into the DHP-insensitive subunit ( , gray segments and thin lines). The detailed amino acid composition is given under “Experimental Procedures.” B, representative of AL12m recorded in the absence (Control) or in the presence of the DHP antagonist (−)-BayK 8644 (10 μM) or the DHP antagonist (−)-isradipine (10 μM). Currents were elicited by depolarization from a holding potential of −80 mV to the peak potential (antagonist) or 10 mV negative to the peak potential (agonist) of the current-voltage relations at a pulse frequency of 0.1 Hz. C, sequence alignments of transmembrane segments IIIS6 and IVS6 from rabbit cardiac muscle ( ), carp skeletal muscle ( ), and ( ) body wall muscle ( ) L-type calcium channel subunits. Amino acid divergences used for our point mutations are boxed. Asterisks indicate L-type residues previously identified to be involved in DHP interaction. D, sensitivity of AL12m mutants to 10 μM solutions of the DHP antagonist (−)-isradipine. E, Val to Met conversions in segments IIIS6 and IVS6 confer agonist sensitivity to the resulting AL12m mutants, whereas the Ser to Ala substitution in IVS6 was without effect. The modulation of by 10 μM (−)-BayK 8644 is illustrated. Currents were recorded before (Control) and after application of drug. Capacitative transients were truncated for presentation. One representative recording of at least three experiments is shown.
the specificity of drug-receptor interactions and was unequivocally present in α1A,DHP. As shown in Fig. 5A, (+)-isradipine displayed a clearly higher potency than (−)-isradipine in blocking I_{Ba} of α1A,DHP. In addition, block of α1A,DHP by (−)-isradipine was voltage-dependent. DHP antagonists preferentially bind to the inactivated state of L-type channels, exhibiting a higher affinity at depolarized holding potentials (10), which is observed as an antagonist-induced shift of the steady-state inactivation curve to more negative voltages. Under control conditions, α1A,DHP exhibited steady-state inactivation with a V_{1/2} of −49.5 ± 0.9 mV (n = 3), indistinguishable (p > 0.01) from the −52.4 ± 3.4 mV (n = 3) obtained for α1A,DHP. In the presence of 0.1 μM (−)-isradipine, the V_{1/2} of α1A,DHP was shifted 10.0 ± 1.8 mV (n = 3) in the hyperpolarized direction (Fig. 5B), clearly demonstrating a state dependence of the antagonist effect.

Previous studies have demonstrated that DHP agonist modulation of L-type calcium channels is voltage-dependent (32–34). As shown in Fig. 5C, this feature was observed for the modulation of α1A,DHP by (±)-BayK 8644. At a pulse frequency of 0.1 Hz and a holding potential of −60 mV, the extent of peak I_{Ba} stimulation by 10 μM (±)-BayK 8644 was significantly (p < 0.05) reduced to 1.5 ± 0.1-fold (n = 3) (Fig. 5C, lower panel) when compared with the 3.9 ± 0.5-fold (n = 5) stimulation at a holding potential of −80 mV (Fig. 5C, upper panel). These results demonstrate that the P/Q-type channel mutant α1A,DHP exhibits features of DHP agonist and antagonist modulation that are usually observed for wild-type L-type channels.

Sensitivity of α1A,DHP and α1A,DHP to Other L-type Calcium Channel Modulators—The construction of a DHP interaction domain within the sequence of the DHP-insensitive α1A subunit could possibly impose restrictions on structurally diverse compounds that modulate the resulting mutant channels. Therefore, we tested the response of α1A,DHP and α1A,DHP to the enantiomers of the benzoxadiazol-DHP-(202–791) as well as the structurally unrelated L-type calcium channel agonist FPL 64176. Similar to the modulation by (±)-isradipine and (±)-BayK 8644, α1A,DHP was less sensitive to these calcium channel drugs than α1A,DHP (Fig. 6). Peak I_{Ba} inhibition by 10 μM solutions of the antagonist (−)-(202–791) at test potentials corresponding to the peak of the current-voltage relations was 30 ± 4% (n = 4) for α1A,DHP, which is significantly lower (p < 0.01) than the 64 ± 3% (n = 3) inhibition determined for α1A,DHP (Fig. 6, left column). As for (±)-BayK 8644, a stimulation of peak I_{Ba} by 10 μM solutions of the agonists (±)-(202–791) and FPL 64176 was almost absent in α1A,DHP (Fig. 6A), whereas agonist effects on macroscopic inactivation were again detected (Fig. 6A). The introduction of Met^{1188} also increased the response to these agonists (Fig. 6B), since the stimulation of α1A,DHP-mediated peak I_{Ba} was 1.5 ± 0.1-fold (n = 3) by 10 μM (±)-(202–791) and 2.8 ± 0.5-fold (n = 3) by 10 μM FPL 64176, which is comparable with the extent previously observed for the modulation of other DHP-sensitive chimeric or mutant channels (18, 19).

DISCUSSION

Transfer of DHP Sensitivity in a Gain-of-function Approach—We demonstrate that the high affinity DHP interaction domain of L-type calcium channels can be transposed to the DHP-insensitive α1A subunit by simultaneous conversion of as few as nine amino acid residues to their L-type counterparts. The involvement of eight of these nine L-type residues in DHP interaction has previously been shown (19–21). The importance of an additional L-type methionine residue (Met^{1188}, α_{TC} numbering) to fully support DHP sensitivity represents a new finding presented in this study. The role of Met^{1188} was uncovered by the differences in DHP agonist and antagonist sensitivity of the chimeric calcium channel α_{1b} subunit AL12m, that was constructed by merging sequence stretches from the DHP-insensitive α_{1c} subunit (22) and the α_{1M} subunit, which had been cloned from M. domestica body wall muscle (24). AL12m, in addition to having provided an opportunity to identify the importance of Met^{1188} for DHP interaction, enabled us to characterize for the first time the DHP sensitivity of an ancestral L-type α_{1b} subunit (Fig. 2).
The validity of a gain-of-function approach in the examination of the structural requirements for calcium channel modulation by DHPs was demonstrated by the P/Q-type calcium channel-derived mutant $\alpha_{1A}$ subunit $\alpha_{1A}$-DHP. The simultaneous mutations Y1393T, M1397Q, F1504I, F1507I, V1512M, I1804Y, F1805M, M1811I, and L1812I ($\alpha_{1A}$ numbering) generated full sensitivity for the DHP agonists and antagonists ($6$)-BayK 8644 and ($6$)-isradipine, respectively (Fig. 3), the optical enantiomers of the DHP-(202–791), and even the structurally unrelated benzoyl pyrrole FPL 64176 (Fig. 6). It is important to point out that in addition to these residues a tyrosine (Tyr1503; $\alpha_{1A}$ numbering) and possibly other residues that are conserved between $\alpha_{1A}$ and L-type calcium channel $\alpha_1$ subunits may also participate in the formation of this binding domain (20). $\alpha_{1A}$-DHP closely resembled native L-type channels with respect to DHP modulation. The typical hallmarks of L-type channel modulation, such as stereoselectivity and a shift of the steady-state inactivation curve to more negative voltages by the antagonist as well as the voltage dependence of agonist effects (32–34), were exquisitely preserved in $\alpha_{1A}$-DHP (Fig. 5). Half-maximal inhibition of peak $I_{Ba}$ by the DHP antagonist ($6$)-isradipine (Fig. 4B) occurred with an even higher apparent potency than that reported for recombinant L-type channels expressed in Xenopus oocytes (18). Since DHP antagonists bind with high affinity to the inactivated state of L-type channels (10), the pronounced antagonist sensitivity of $\alpha_{1A}$-DHP...
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Implications for Channel Structure—The L-type residues that successfully transferred DHP sensitivity are located within transmembrane segments IIIS5, IIIS6, and IVS6 (Fig. 7). These regions appear to be particular hot spots of voltage-dependent calcium channels with respect to drug interaction, since they also contain the molecular determinants for phenylalkylamines and benz(othi)azepines (35, 36) and benz(othi)azepines even share Tyr^1490 and Ile^1497 (α1C-a numbering) in transmembrane segment IVS6 as common interaction residues. These results are consistent with findings from in vitro binding studies employing fluorescent calcium channel ligands and photoaffinity labeling (37), which demonstrated that the DHP and benz(othi)azepine binding domains are localized in close proximity to each other on the L-type α1 subunit. At least some of the residues that interact with these drugs seem to face the channel pore. This has previously been demonstrated for Tyr^1490 by an increase of N-methyl-D-glucamine conductivity following a Tyr to Ala conversion (35). To bridge the DHP molecule, the position of the interaction residues must be within a distance of approximately 13–15 Å (assuming a maximal drug diameter of about 9 Å and bond lengths of 2–3 Å). Accordingly, transmembrane segments IIIS5, IIIS6, and IVS6 must lie in close vicinity to each other in the folded α1 subunit, with the DHP binding domain oriented toward the channel pore.

Transmembrane segments of voltage-gated ion channels are predicted to display an α-helical structure (39, 40). Based on this model, all of the identified L-type residues interacting with DHPs align to the same side of the respective α-helices, with the exception of Met^1188. This could indicate a more indirect effect of Met^1188, mediated by changes in electrophysiological properties that result in an increase of DHP sensitivity. However, biophysical parameters that could affect DHP sensitivity, such as steady-state inactivation as well as I_{Ba}, activation, were not appreciably altered among the mutant channels α1A-DHPi and α1A-DHP. We therefore assume that a direct contribution of binding energy by Met^1188 is responsible for its importance in DHP interaction. Since this residue is located on the opposite side of Tyr^1179 and Ile^1180 (α1C-a numbering) in a putative α-helix, this can only be accomplished if segment IIIS6 protrudes deeply into the pore. Alternatively, and similar to the S6 segment of Shaker potassium channels (40), portions of the cytoplasmic half of IIIS6 may be tilted or deviate from a straight α-helix. A specific example for this hypothesis is a proline (Pro^1508, α1C-a numbering), that is conserved in non-L-type α1 subunits and therefore present in the IIIS6 segment of α1A-DHP. This residue separates Met^1188 from the Tyr^1179-Ile^1180-Ile^1183 DHP interaction motif and replaces an alanine (Ala^1184, α1C-a numbering) that is conserved throughout L-type α1 subunits. In analogy to the model of potassium channel S6 segments (40), this proline residue could produce a tilt in the putative α-helix of transmembrane segment IIIS6. As a result Met^1188 might achieve a more favorable orientation for drug interaction in α1A-DHP than in native L-type channels, which could in part explain the remarkably high DHP sensitivity of this mutant. The consequences of a Pro to Ala conversion in transmembrane segment IIIS6 of α1A-DHP on functional DHP modulation and radioligand binding will challenge this hypothesis.

In conclusion, the gain-of-function mutant α1A-DHP represents a valuable tool to investigate the structure-activity relationship between DHPs and the molecular determinants of their interaction domain. Expression in mammalian cells and radioligand binding studies as well as electrophysiological analysis of the resulting mutants will provide a suitable assay for an even more refined study. Further characterization of the structural determinants of channel gating is under way and, together with our findings, will eventually unravel the molecular mechanism of channel modulation by DHPs. Elucidation of the structure-activity relationship between α1A-DHP and DHPs will provide useful molecular information that may be exploited to design and develop compounds that selectively interact with non-L-type calcium channels. Such drugs could be of therapeutic value for the treatment of mental disorders, stroke, and pain (41). Most recently, certain forms of migraine and ataxia have been linked to mutations in the α1A subunit gene (42, 43), which are expected to result in altered channel activity. Selective drugs, therefore, may also be effective in the treatment of diseases associated with changes in PVQ-type channel function.

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