Molecular Studies on the Voltage Dependence of Dihydropyridine Action on L-type Ca\(^{2+}\) Channels

CRITICAL INVOLVEMENT OF TYROSINE RESIDUES IN MOTIF IIIS6 AND IVS6*

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The interaction site(s) of dihydropyridine (DHP) antagonists and agonists have been identified by site-directed mutagenesis and localized on motifs IIIS6, IIIIS6, and IVS6 of L-type voltage-gated calcium channels. In this study, we investigated the voltage-dependent action of DHPs with mutants of the IIIS6 and IVS6 segments of a cardiac calcium channel. Tyrosine residues in both motifs (Tyr\(^{1178}\) and Tyr\(^{1489}\)) strongly contributed to the action of DHP agonists and antagonists. When these two sites were mutated, the communication between the voltage sensor and the DHP interaction site(s) was substantially impaired. In contrast, mutants of a nearby Ile (Ile\(^{1182}\)) had much less influence on DHP agonist and antagonist interaction, and the voltage dependence of DHP antagonists was very similar to that of the wild type. The effect of a mutating of Ile\(^{1182}\), on agonist or antagonist action, however, depended strongly on the type of amino acid change. When Ile\(^{1182}\) was substituted with alanine, small changes were noted for DHP agonist and antagonist action. Changing this site into phenylalanine, however, significantly decreased the action of the DHP antagonist. These data show that Ile\(^{1182}\) can preferentially interact with DHP antagonists, but has a lesser contribution in agonist interaction. Thus, even though the agonist and antagonist interaction sites for DHPs with L-type calcium channels may overlap, some amino acids in this site may exhibit a preference for either DHP enantiomers.

High voltage-gated calcium channels (HVGCC) play a key role in excitable cells in coupling membrane potential changes to biological activity such as contraction, secretion, and neurotransmitter release (1–3). The general architecture of HVGCCs is that of a heterotetrameric polypeptide complex \(\alpha_1, \alpha_2, \beta,\) and \(\gamma\) (4, 5). Based on pharmacological and electrophysiological criteria, the HVGCCs are divided into five subclasses, L, N, P, Q, and R (4–6), and the distinguishing phenotypes are carried by the \(\alpha_1\) subunit, which contains the voltage sensor, the channel pore (7, 8), and high affinity binding sites for at least three classes of organic calcium channel blockers; 1,4-dihydropyridines (DHPs), phenylalkylamines, and benzothiazepines. Photoaffinity labeling results show that DHPs, phenylalkylamines, and benzothiazepines bind to motif IIIS6 and IVS6 (10–12, 14), IVS6 (9), and IIIS6 and IVS6 (14, 22), respectively. A further confirmation of the binding site of 1,4-dihydropyridines arose from a chimera approach (13). The DHP sensitivity of the \(\alpha_1C\) subunit was lost when part of the IVS6 segment was replaced by the corresponding but not totally equivalent portion of the DHP-insensitive \(\alpha_{14}\) subunit (25). This approach also showed that part of the IIIS6 transmembrane domain previously noted as a “primary site” for DHP photoaffinity labeling (10, 11) was not involved in the DHP receptor. However, this study revealed that “different” sections of IVS6 seem to be involved in DHP agonist action and DHP antagonist action (13). A similar strategy using “constructive chimeras” between \(\alpha_{1C}\) and \(\alpha_{14}\) identified IIIS5, IIIS6, the connecting linker segment as well as IVS5-IVS6 and part of the IVS6 as elements of the DHP binding site (15). This work also agreed with the data of our laboratory (13) in that different portions of IVS6 appear to be involved in DHP agonist and antagonist binding action (15).

Further, recent advances using a combination of chimera approach and site-directed mutagenesis revealed individual amino acids that may be critical for DHP binding (16, 17). Peterson et al. (16) found that the outermost 60% of the IIIS6 and IVS6 transmembrane segments of \(\alpha_{15}\) are critical determinants of high affinity DHP binding. A tyrosine and two isoleucine residues in IIIS6 arranged in a YM(\(\alpha\)I)\(_5\) sequence in IVS6 were found to contribute to the DHP receptor site. Interestingly, in motif IIIS6, the important tyrosine residue believed to be involved in DHP action is conserved between DHP-sensitive and DHP-insensitive channels. A somewhat

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* The abbreviations used are: HVGCC, high voltage-gated calcium channel; DHP, dihydropyridine; HP, holding potential; (+)R-202–791, (−)R-4(2,1.3-benzoazindol-4-yl)1.4-dehydro-2.6-dimethyl-5-nitro-3-pyridinecarboxylic acid isopropyl ester; (−)S-202–791, (+)S-4(2,1.3-benzoazindol-4-yl)-1.4-dihydro-2.6-dimethyl-5-nitro-3-pyridine-carboxylic acid isopropyl ester.
similar finding was made for the cardiac α_{1C} subunit (17), although in the latter only IVS6 was thoroughly tested by mutagenesis studies, and the amino acids that contribute to the DHP interaction site are almost identical with those published by Peterson et al. (16), with the exception that these authors (17) attributed less importance to the tyrosine residues.

Recent studies (19, 20) point to the importance of the IIIS5 motif in the contribution of DHP sensitivity in cardiac calcium channels. Mutation of a threonine residue in IIIS5 (Thr1066) into tyrosine (which is a natural substitution in the α_{1A} "P/Q-type channel") rendered the construct totally insensitive to DHP agonist and antagonist action (19, 20). Similar to the functional electrophysiological studies, no DHP antagonist radioligand binding was detected with this mutant (20). Further, it has been shown that Thr^{1066} and Glu^{1070} are not only crucial for DHP sensitivity but also display different effects for DHP agonist and antagonist action. Thr^{1066} appears to be involved in mediating the DHP antagonist function, whereas the DHP agonist function requires the presence of both Thr^{1066} and Glu^{1070} (19).

Altogether, these results show the involvement of a number of amino acid residues in the IIIS5, IIIIS6, and IVS6 transmembrane segments of the cardiac calcium channel in the formation of the DHP binding/action site. These data, of course, do not eliminate the involvement of other motifs.

The extent of DHP block of the L-type calcium channel depends on the holding potential, becoming more "efficient" in the inactivated state (27–29), which is represented by more depolarized potentials. The studies of Peterson et al. (16) were done at zero membrane potential using radioligand DHP binding. The work of Schuster et al. (17) employed electrophysiological methodologies; therefore, it is possible that different experimental conditions may account for the discrepancies; i.e. in one case binding to membranes yields accurate K_v values; in the other case, electrophysiological data provide relative pharmacological "potency," and the two may not be the same. These findings prompted us to investigate point mutants of α_{1C} in IIIS6 and IVS6, required for high affinity interaction of DHPs, with a special emphasis on voltage dependence of DHP action.
EXPERIMENTAL PROCEDURES

Mutagenesis and in Vitro Synthesis of cRNAs—The full-length (30, 31), rabbit heart L-type channel α₁ subunit cDNA (Ca9) (13) was constructed in the plasmid pBluescript SK(-). The mutations were introduced by the polymerase chain reaction method. Oligonucleotides encoding Pm1, Io rEcoRV sites and carrying the designed base mismatches served as forward primers, and oligonucleotides having NsiIo rBstEII sites as reverse primers. The polymerase chain reaction products were ligated into and replaced the corresponding regions of wild type α₁ cDNA. The whole regions replaced by polymerase chain reaction products were sequenced, and the presence of the desired mutations was verified. cRNAs for wild type α₁ subunit, mutant α₁ subunits, skeletal muscle Ca²⁺ channel α₂δ subunit (32), and human heart β₃ subunit (33) were synthesized by in vitro transcription.

Expression of Calcium Channels in Xenopus Oocytes—Xenopus oocyte isolation and cRNA injection was performed as published elsewhere (13). Briefly, female Xenopus laevis (purchased from Xenopus I, Ann Arbor, MI) frogs were anesthetized by exposing them for 15–20 min to 0.15% methanesulfonate salt of 3-aminobenzoic acid ethyl ester (MS-222; Sigma) solution before pieces of the ovary were removed. The follicular layers from isolated oocytes were digested using 2.0 mg/ml collagenase (Type IA; Sigma) dissolved in OR-2 medium containing (in mM): 82.5 NaCl, 1 KCl, 1 MgCl₂, 5 HEPES, pH 7.5. After 1.5–2.0-h digestion, stage V-VI oocytes were selected and incubated at 19 °C in P/S medium containing (in mM): 96 NaCl, 2.0 KCl, 1.0 MgCl₂, 1.8 CaCl₂, 5.0 HEPES, 2.5 sodium pyruvate, and 0.5 theophylline at pH 7.5, and supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin before injection was started.

Oocytes were injected with a total of 50 nl of solution of cRNA from wild type or mutant rabbit heart α₁ subunit (0.2–0.5 μg/ml), in combination with skeletal α₂δ and human heart β₃ subunit cRNAs (0.2 μg/ml each) to enhance the expressed Ca²⁺ currents. The injected oocytes were incubated in P/S solution at 19 °C. Ca²⁺ channel currents were recorded after 2–4 days of injection of the cRNAs at room temperature (20–21 °C) using the standard two-electrode voltage-clamp technique.

The recording medium was a Ca²⁺- and Cl⁻-free solution composed of (in mM): 40 Ba(OH)₂, 50 N-methyl-D-glucamine, 2 KOH, 5 HEPES, 0.5 niflumic acid, pH adjusted to 7.4 with methanesulfonic acid. Voltage

| Table I |
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| The calculated EC₅₀ and IC₅₀ values of wild type and mutant channels for (+)-S-202–791 and (-)-R-202–791 |
| HP | Wild type | Y1178A | I1182A | I1182F | Y1489I |
| --- | --- | --- | --- | --- | --- |
| mV | (+)-S-202–791 | (-)-R-202–791 |
| 30 | 0.32 | 11.10 | 0.56 | 0.33 | 8.44 |
| 80 | 0.43 | 12.50 | 0.85 | 0.64 | 9.03 |

FIG. 2. Effects of (+)-S-202–791 on Ba²⁺ current through wild type and mutant Ca²⁺ channels at −30-mV holding potential. A, current traces in the absence (○) and presence (●) of 1 μM (+)-S-202–791 for the wild type and mutant channels Y1178A, I1182A, I1182F, and Y1489I. The current traces that showed the largest amplitude are superimposed. B, concentration-response curves for the effects of (+)-S-202–791. The peak Ba²⁺ current was expressed as the ratio to that without the drug. Data points are mean values from 3–35 experiments. Error bars show the S.E. (shown only for the wild type for clarity).
and current electrodes were filled with 3 M KCl and had a resistance of 0.5–1.5 megohms. Currents were recorded using an Axoclamp-2A (Axon Instruments Inc., Foster City, CA) amplifier. Whole cell leakage and capacitive currents were subtracted on line using the P/4 procedure. Currents were digitized at 1 kHz after being filtered at 1 kHz. The pClamp software (version 5.5) was used for data acquisition and analysis. Some of the oocytes showed significant rundown during normal recording conditions and oocytes that showed only less than 15% rundown over the first 3 min were included in the analysis. The DHP agonist (−)-R-202–791 and antagonist (−)-R-202–791 were dissolved (10 mM) in ethanol and diluted to the final concentration in Ba2+ recording solution. Ethanol alone at final concentration of 0.1% did not have any effect on expressed Ba2+-currents. The endogenous oocyte Ca2+-activated Cl− current was suppressed by injection of 50 nl of a solution containing 100 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid and 1 M Tris (pH 7.4) 2 h before the recording was made (34). The drug-containing solution was applied directly to the experimental chamber. Concentration-response curves were obtained from measurements of peak inward Ba2+-currents in the control solution and 1 min after changing the bath solution to different DHP concentrations. Ba2+-currents were elicited by a 1400-ms long depolarizing pulse from a holding potential of −80 mV or −30 mV to test potentials between −20 mV and 40 mV in 10-mV increments. Statistical significance was analyzed using the Student’s t test (p < 0.05). Data are expressed as means ± S.E.

RESULTS

Influence of Point Mutations on the Sensitivity to a DHP Agonist—Previous reports (16, 17, 19, 20) have identified a number of amino acid residues in IIIS5, IIIS6, and IVS6 of L-type calcium channels that contribute to the interaction of DHPs. Action of DHP antagonists strongly depend on the conformation of the channel in the electric field of the membrane. We have assumed that there would be amino acids, belonging to the DHP interaction site, that would exert a major contribution to the voltage dependence of DHP action. Therefore, we investigated the contribution of some point mutations in IIIS6 and IVS6 in the voltage dependence of DHP agonist and antagonist action. Wild type and mutant calcium channels were expressed in Xenopus oocytes, the oocytes were voltage-clamped at holding potentials (HPs) of either −30 or −80 mV, and inward Ba2+-currents were elicited by depolarizing step pulses to various potentials. At HP −80 mV, the peak IbA of the...
wild type, Y1178A, I1182A, I1182F, and Y1489I channels were -489.4 ± 39.3 nA (n = 48), -470.3 ± 69.1 nA (n = 32), -583.2 ± 93.6 nA (n = 15), -477.1 ± 28.3 nA (n = 42), and -355.7 ± 36.4 nA (n = 62), respectively (Fig. 1A). Application of DHP agonist, (+)-S-202–791 (1 μM) to the oocytes expressing the wild type channel increased the peak current amplitude 3.3 ± 0.3-fold (n = 18) at HP -80 mV (Fig. 1, A and B) and 3.6 ± 0.3-fold (n = 12) at HP -30 mV (Fig. 2, A and B). The EC50 values for the wild type channel at -80 and -30 mV HP were 0.32 and 0.43 μM, respectively (Figs. 1B and 2B). Mutants of Ile1182 in motif III, I1182A, and I1182F, showed virtually the same response as the wild type channel at both HPs, whereas Y1489I and Y1178A exhibited a significantly diminished response to (+)-S-202–791 at both HPs. The difference in the EC50 was less than 2-fold for mutants of Ile1182 and showed no significant variation at different HPs (Table I). In contrast, the other two mutants, Y1489I and Y1178A, showed decreased sensitivity to (+)-S-202–791. Motif III mutant Y1178A showed a 35-fold (HP -30 mV) and 29-fold (HP -80 mV) increase in the EC50 compared with that of the wild type, and those for motif IV mutant Y1489I were 26-fold (HP -30 mV) and 21-fold (HP -80 mV) (Table I) higher, respectively, compared with the wild type. These results suggest that Tyr1178 and Tyr1489, but not Ile1182, are critical for the action of DHP agonists. In addition, the effect of the DHP agonist was not dependent on the holding potential, which is in good accordance with the literature (27–29).

Influence of Point Mutation on the Sensitivity to a DHP Antagonist—Application of the DHP antagonist, (+)-R-202–791 (1 μM) to oocytes expressing wild type channels decreased the peak current amplitude to 78.8 ± 3.3% (n = 18) at HP -80 mV (Fig. 3A) and 35.0 ± 4.0% of the control (n = 8) at HP -30 mV (Fig. 4A). The IC50 values were 0.12 and 2.53 μM, respectively. When using the HP -30 mV, I1182A showed almost identical sensitivity as in the wild type, whereas other mutants showed decreased sensitivity (Fig. 4B). The IC50 values of I1182F, Y1178A, and Y1489I were 8.5, 31, and 18 times higher than that of the wild type (Table I). It is of interest that only when Ile1182 was replaced by Phe, but not by Ala, the sensitivity of the channel to (+)-R-202–791 was substantially decreased. Because maximal inhibition could not be attained under a -80 mV HP, the differences between wild type and

FIG. 4. Block of Ba2+ current through the wild type and mutant Ca2+ channels by (+)-R-202–791 at -30-mV holding potential. A, current traces in the absence (○) and presence (●) of 1 μM (+)-R-202–791 for the wild type and mutant channels Y1178A, I1182A, I1182F, and Y1489I. The current traces that showed the largest amplitude were superimposed. B, concentration-response curves for the effects of (+)-R-202–791. The peak Ba2+ current was expressed as the ratio to that without the drug. Data points are mean values from 3–20 experiments. Error bars show the S.E. (shown only for the wild type for clarity).
mutants were less evident. The IC50 values of Y1178A, I1182A, I1182F, and Y1489I were 5.1, 2.6, 8.2, and 5.7 times higher than that of the wild type. In this case, I1182A showed a slightly decreased sensitivity (Table I).

Mutation Affects the Voltage Dependence of Activation and the Modulation by a DHP Agonist—Representative current-voltage relationships for the wild type and mutant channels are shown in Fig. 5. Interestingly, the two Ile1182 mutants showed an altered potential of the maximum peak I Ba compared with the wild type in control conditions. Peak voltage was shifted to a positive direction in I1182A and to a negative direction in I1182F. The shifts were statistically significant (p < 0.05) compared with the wild type. These phenomena were observed at both HPs (Fig. 5, A and B). The peak voltage was not altered in Y1178A and Y1489I (Fig. 5, Table II). After applying 1 μM (+)-S-202–791, the negative shift of peak voltage was significantly smaller (p < 0.05) in Y1178A and Y1489I than that of the wild type, whereas Ile1182 mutants were not different from the wild type. In fact, no significant peak voltage shift was observed with Y1178A, after treatment with 1 μM (+)-S-202–791. These tendencies are almost parallel to the effects of (+)-S-202–791 on the peak current amplitude.

**Fig. 5.** The current-voltage relationships were measured with 1400-ms voltage steps from the HP of −30 mV to the indicated potentials. A, peak current was recorded in the absence (○) and presence (●) of 1 μM (+)-S-202–791. B, peak currents recorded in the absence (○) and presence (●) of 1 μM (−)-R-202–791.
The question arises whether the diminished peak-voltage shift of mutants Y1178A and Y1489I in response to 1 μM (+)-S-202–791 is due to decreased affinity to the drug, i.e., at isoeffectice concentrations of the drug (the drug concentration that causes identical peak amplitude increase for different mutants and for control as well) or whether mutants Y1178A and Y1489I show a decreased peak amplitude shift even at saturating concentrations of (+)-S-202–791. Testing all the mutants at various concentrations of (+)-S-202–791 showed that the latter was the case (Fig. 6). The wild type and I1182A and I1182F exhibited a concentration-dependent peak amplitude increase (Figs. 1B and 2B) and negative shift of peak voltage. The shift of peak voltage saturated at about 1 μM (+)-S-202–791 and reached a −17–19 mV value (Fig. 6). On the contrary, mutants Y1178A and Y1489I reached a maximal shift of peak voltage at about 30 and 5 μM (+)-S-202–791, respectively. The maximal peak voltage shift for these latter two mutants was at about −8–12 mV, which is considerably lower than that observed for the wild type and mutants of I1182.

The Influence of Mutations on Inactivation-accelerating Effects of (−)-R-202–791—To analyze inactivation kinetics, we employed “the maximal slope of decay” as the parameter of inactivation. The value of the maximal slope of decay was normalized by the current amplitude at a particular time since the slope is thought to be proportional to the current (Fig. 7A). We observed no significant differences in inactivation kinetics between the wild type and two Le1182 mutants. The maximal slope of decay of the wild type was 0.606 ± 0.074 ms⁻¹ (HP −30 mV, n = 28), whereas those of I1182A and I1182F were 0.567 ± 0.044 ms⁻¹ (n = 10) and 0.565 ± 0.067 ms⁻¹ (n = 37), respectively. In contrast, Y1178A and Y1489I showed significantly faster inactivation. The maximal slope of decay of Y1178A and Y1489I were 0.920 ± 0.091 ms⁻¹ (n = 9, p < 0.05) and 1.193 ± 0.087 ms⁻¹ (n = 31, p < 0.05), respectively. The concentration-response relationship of (−)-R-202–791 on inactivation kinetics (HP −30 mV) is depicted in Fig. 7B. Although I1182F, Y1178A, and Y1489I showed decreased sensitivity to (−)-R-202–791 regarding the current amplitude, all mutants but Y1178A had virtually the same sensitivity to the compound in terms of acceleration of inactivation.

**DISCUSSION**

Several point mutations (Y1178A, I1182A, I1182F, and Y1489I) in IIIS6 and IVS6 transmembrane segments of the cardiac calcium channel were tested for their involvement in

**TABLE II**

| HP        | Wild type | Y1178A | I1182A | I1182F | Y1489I |
|-----------|-----------|--------|--------|--------|--------|
| −30 mV    | 20.0 ± 0.9 | 20.1 ± 1.3 | 25.0 ± 1.0<sup>a</sup> | 15.4 ± 0.6<sup>a</sup> | 20.8 ± 0.8 |
| Voltage shift | −16.1 ± 1.4<sup>b</sup> | −2.2 ± 1.4<sup>b</sup> | −12.2 ± 1.0<sup>b</sup> | −15.6 ± 1.4<sup>b</sup> | −7.6 ± 0.6<sup>b</sup> |
| −80 mV    | 20.2 ± 0.8 | 21.2 ± 1.6 | 25.3 ± 0.4<sup>a</sup> | 16.9 ± 1.5<sup>a</sup> | 21.9 ± 0.5 |
| Voltage shift | −17.2 ± 1.6<sup>b</sup> | −0.9 ± 2.1<sup>b</sup> | −11.8 ± 1.2<sup>b</sup> | −13.4 ± 1.6<sup>b</sup> | −8.3 ± 1.0<sup>b</sup> |

<sup>a</sup> Significantly different (p < 0.05) versus wild type.
<sup>b</sup> Significantly shifted from control after the application of 1 μM (+)-S-202–791 (p < 0.05 versus 0).
the well known voltage-dependent action of dihydropyridines. Mutations Y1178A and Y1489I had a great influence on the agonist action of (+)-S-202–791 at a holding potential of −80 or −30 mV. The ECSD increased by a factor of 29–35-fold for both of these mutations, whereas the voltage dependence of agonist action was virtually the same for these mutations. Mutations of Ile1182 of the cardiac calcium channel either to Ala or Phe had only a minor impact on the agonist action of (+)-S-202–791.

The holding potential had a more prevailing effect on the antagonist action of (+)-R-202–791 when the same mutants were tested. Characteristically lower ICSD values were observed for all mutants at −30-mV holding potential than at −80-mV holding potential. Similarly, more dramatic changes in ICSD values were noted for Y1178A and Y1489I than for mutants of Ile1182 as compared with the wild type channel. However, the substitution of I1182A and I1182F showed distinctly different behaviors upon antagonist application. The ICSD of I1182A at −30-mV holding potential remained essentially the same as that of the wild type while there was an 8.6-fold increase in the ICSD when Ile1182 was substituted for phenylalanine. This change remains the same at a more negative holding potential (−80 mV) when compared with that of the wild type.

The work of Grabner et al. (15) and Mitterdorfer et al. (19) showed that amino acids in III5 are responsible for exerting differential effects toward DHP agonist and antagonist action. Specifically, the presence of Thr1006 (Thr1006 according to the nomenclature of Mitterdorfer et al. (19)) mediated a substantial portion of the DHP antagonist action when this amino acid was introduced into the α1A non-DHP-sensitive calcium channel. However, this mutant construct showed no DHP agonist sensitivity. To render an α1,, non-DHP-sensitive calcium channel, sensitive to the agonist, the combined presence of Thr1006 and Glu1010 (Thr1006 and Glu1070 according to the numbering of Mitterdorfer et al. (19)) was necessary (19). Based on a number of previous observations (13, 15, 19, 20) and on our present work, it is now clear that, even though sites might overlap, some amino acids (Thr1006 and Glu1010) preferentially contribute to the antagonist and agonist interaction sites, respectively. Further, distinct substitutions at other sites (Ile1182) may induce conformational changes that preferentially favor the agonist or the antagonist interaction.

The observation that substitution of Ile1182 for phenylalanine decreased the sensitivity to antagonist without affecting the agonist sensitivity implies that a distinct site of action, and/or favorable conformation of the protein exists even though these site(s) may partly or completely overlap. This observation is, in principle, in accordance with a previous report from our laboratory (13) and contradicts that of Peterson et al. (16), who claimed that the agonist and antagonist binding sites totally overlap, and especially for Ile1182 no discriminative function was noted between the agonist and antagonist action. This latter work, however, utilized an experimental system that is significantly different from ours; it uses skeletal muscle α1A subunit mutants and the conclusions are based only on direct radioligand binding to membranes which of course are at 0-mV membrane potential.

We have investigated the inactivation kinetics of some DHP interaction site mutants. Due to the potential influence of contaminating Cl− currents in oocytes we used the “maximal slope of decay” as the parameter of inactivation. The Ile1182 mutants showed maximal slope of decay very close to that of the wild type. Surprisingly, the Y1178A and Y1489I exhibited significantly faster inactivation than the wild type. Similar findings were reported by Shuster et al. (17); however, these authors attributed the increased current decay to the presence of the antagonist, and they raised the possibility that this phenomenon is a consequence of a second, lower affinity binding site. Mutations of the tyrosine in rat brain α1,, (18) that correspond to Tyr1489 of the rabbit heart channel caused changes of the apparent reversal potential of peak calcium channel current and also showed changes in permeation properties of the channel, suggesting that this amino acid faces the channel pore and is involved in the ion-conducting pathway. Here we have shown that the mutants we investigated exhibited differential properties to the action of the antagonist (−)-R-202–791 in terms of accelerating inactivation. In this regard, wild type, mutants of Ile1182 and Y1489I fell in the same category; i.e. the antagonist compound (−)-R-202–791 inhibited the current amplitude to various extents; however, the concentration dependence of the drug on the acceleration of inactivation appeared virtually identical. Contrary to this, the sensitivity of the inactivation of Y1178A in response to (−)-R-202–791 showed altered concentration dependence compared with the previous group of mutants. This suggests that in these mutants, although the effect of the DHP antagonist in blocking the peak current is lowered, its effect on acceleration inactivation kinetics remains unchanged. In other words, distinct sites of action of DHP antagonists might exist for blocking the peak current and for accelerating the inactivation of the channels.

A closer examination of the concentration-response relationship of (+)-S-202–791 on the shift of peak potential showed that mutants of Ile1182 overlap with that of the wild type resulting in a negative shift of about 16–18 mV. On the other hand, the shift of peak potential for Y1178A and Y1489I differed about 10-fold, and the maximum shift for these mutants appeared around −10 mV. The interaction of DHP agonists with L-type calcium channels increase the probability of opening and the mean open time (35). In terms of kinetics, these drugs shift the channel activation to hyperpolarizing potentials. The structural basis for this effect is largely unknown, however, a previous model, that assumed that the DHP binding site is in close proximity to the S4 segments (36) is doubtless since recent findings have indicated that amino acids in III5, III6, and IVS6 transmembrane segments contribute to the DHP interaction site (16, 17, 19, 20). Our results indicate that mutation of Tyr1178 and Tyr1489 largely impaired the communication between the S4 voltage sensor region and the DHP interaction site, resulting in a partial loss of the voltage shift by a DHP agonist. In contrast, mutations of Ile1182 assuming an α-helical structure for the III6 segment, is just one turn away from Tyr1178 and has no influence on the communication between the S4 segment and the DHP interaction site.

The structural rearrangement of voltage-gated calcium channels that “moves charges” in the electric field of the membrane in response to depolarization is a well documented phenomenon (37–39); however, the exact molecular movements of the channel protein are highly speculative (40, 41). Recent molecular evidence for the Shaker potassium channel (42) showed that during channel activation a stretch of seven amino acids of the S4 region moved from a buried position into the extracellular environment. Considering the analogous structure and activation mechanism, there are a number of reasons that the behavior of all voltage-gated calcium channels is similar. Thus, it is likely that the movement of the outer segment of the S4 induces movements of the outer part of the III6 and IVS6 segments.

At the present time, 13 amino acid substitutions in III5, III6, and IVS6 have revealed regions involved in DHP interaction (16, 17, 19, 20), with 9 out of 13 substitutions showing significant effects on DHP interaction (e.g. Kd to DHP antagonist increased at least 5-fold). A question remains as to
whether all of these amino acid side chains actually interact with the small DHP molecule, or whether only a few provide direct interaction areas, and the remainder of the amino acid side chains possibly function as “conformation stabilizers” for the α4 protein.

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