Serine Residue in the IIIS5-S6 Linker of the L-type Ca\(^{2+}\) Channel \(\alpha_{1C}\) Subunit Is the Critical Determinant of the Action of Dihydropyridine Ca\(^{2+}\) Channel Agonists*

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The dihydropyridine (DHP)-binding site has been identified within L-type Ca\(^{2+}\) channel \(\alpha_{1C}\) subunit. However, the molecular mechanism underlying modulation of Ca\(^{2+}\) channel gating by DHPs has not been clarified. To search for novel determinants of high affinity DHP binding, we introduced point mutations in the rat brain Ca\(^{2+}\) channel \(\alpha_{1C}\) subunit (rbCII or Ca\(_{1,2c}\)) based on the comparison of amino acid sequences between rbCII and the ascidian L-type Ca\(^{2+}\) channel \(\alpha_{1}\) subunit, which is insensitive to DHPs. The \(\alpha_{1C}\) mutants (S1115A, S1146A, and A1420S) and rbCII were transiently expressed in BHK6 cells with \(\beta_{1A}\) and \(\alpha_{1}\) subunits. The mutation did not affect the electrophysiological properties of the Ca\(^{2+}\) channel, or the voltage- and concentration-dependent block of Ca\(^{2+}\) channel currents produced by diltiazem and verapamil. However, the S1115A channel was significantly less sensitive to DHP antagonists. Interestingly, in the S1115A channel, DHP agonists failed to enhance whole-cell Ca\(^{2+}\) channel currents and the prolongation of mean open time, as well as the increment of NP\(_i\). Responsiveness to the non-DHP agonist FPL-64176 was also markedly reduced in the S1115A channel. When S1115 was replaced by other amino acids (S1115D, S1115E, S1115F, S1115G, S1115H, S1115I, S1115L, S1115P, S1115Q, S1115R, S1115S, S1115T, or S1115V), only S1115T was slightly sensitive to DHPs. The \(\alpha_{1C}\) subunit, which is insensitive to DHPs, the high affinity binding pocket of DHPs. Indeed, alanine-scanning mutagenesis of IIS6 and IVS6 showed that some amino acids conserved in both high affinity binding site of DHPs.

Voltage-dependent L-type Ca\(^{2+}\) channels play critical roles in shaping action potentials, excitation-contraction coupling, and excitation-secretion coupling in a variety of tissues. L-type Ca\(^{2+}\) channels are composed of at least four subunits, \(\alpha_{1}, \alpha_{1}, \alpha_{1}, \beta(1)\), and \(\beta(1)\). Ca\(^{2+}\) channel \(\alpha_{1}\) subunits are subdivided into five distinct subtypes based on biophysical and pharmacological criteria, i.e. L (\(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\)), \(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\)), \(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\)), N (\(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\))), F/Q (\(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\))), P (\(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\))), and R (\(\alpha_{1}^{\alpha_{1}}\) (Ca\(_{1,2}\))). Classical Ca\(^{2+}\) channel antagonists bind to L-type Ca\(^{2+}\) channel \(\alpha_{1}\) subunits with high affinity (4, 5). Single-channel recording of L-type Ca\(^{2+}\) channel currents showed three distinct modes of Ca\(^{2+}\) channel gating: brief openings (mode 1), no openings due to channel unavailability (mode 0), and long-lasting openings and very broad closing (mode 2) (6). Bay K 8644 enhances Ca\(^{2+}\) channel current by promoting mode 2, whereas nifedipine inhibits the current by favoring mode 0 (6). However, the conformational change accompanied with channel gating and its modulation by Ca\(^{2+}\) channel agonists and antagonists is largely unknown. Determination of amino acids that are critical for the specific interaction with Ca\(^{2+}\) channel agonists and antagonists will help clarify the binding pockets and the molecular mechanism underlying modulation of Ca\(^{2+}\) channel gating.

Experimental Procedures

Point Mutations in rbCII—Single mutations were generated by introducing TuCa1-type amino acids into rat brain \(\alpha_{1C}\) subunit (rbCII, Ca\(_{1,2c}\)), which was kindly supplied by Dr. T. P. Snutch (15). Single point mutations, S1115A, and S1146A, were introduced into IIS5-S6 channel antagonists binding to L-type Ca\(^{2+}\) channel \(\alpha_{1}\) subunits, as a critical determinant of DHP binding and of the action of DHP agonists.

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‡ The abbreviations used are: Bay K 8644, DHP, dihydropyridine; GFP, green fluorescence protein.
linker by replacing the PstI (3384)-XmnI (3743) fragment of rbCII, and A1420S was introduced into IVS5-S6 linker by replacing the PvuII (4185)-XhoI (4607) fragment of rbCII using the QuickChange method (Qiagen). Polymerase chain reaction was performed (30 s at 94 °C, 30 s at 50 °C, and 60 s at 74 °C for 30 cycles) using Pfu polymerase (Stratagene) as confirmed by sequence analysis. All a1C mutants were inserted into the expression plasmid pCDNAII.

Transient Expression of a1C Mutants in BHK6 Cells—Mutated a1C subunits were transiently expressed in BHK6 cells that stably express rabbit β1A and α2δ subunits (16). Dulbecco’s modified Eagle’s medium, containing 5% fetal bovine serum, 30 units/ml penicillin, and 800 μg/ml G418, was used for culture medium. Cells were plated on coverslips coated with 0.5% gelatin and transfected with pCDNAIII-a1C mutants together with GFP (pEGFP-C2) using Superfect (Qiagen). Cells were used for electrophysiological experiments 24–48 h after transfection.

Whole-cell Patch-clamp Recording—The whole-cell L-type Ca2⁺ channel currents were recorded with Ca²⁺ (2 mM) as a charge carrier in bath solution containing (in mM): 137 NaCl, 5.4 KCl, 1 MgCl₂, 10 glucose, 10 HEPES, 2 CaCl₂ (pH 7.4, adjusted with NaOH) at room temperature. The resistance of the heat-polished microelectrodes ranged between 2 and 4 MΩ when filled with the internal solution composed of (in mM): 120 CsCl, 20 tetraethyl ammonium chloride, 14 EGTA, 5 MgATP, 5 disodium creatine phosphate, 0.2 GTP, 10 HEPES, 0.2 L-arginine, and 200 μM GTP (pH 7.3, adjusted with KOH) as measured using a patch/whole-cell clamp amplifier (Nihon Kohden, Tokyo, Japan) or Axopatch 1D (Axon Instruments, Inc., Foster City, CA) via an analog to digital converter (Digidata 1200, Axon Instruments, Inc.). Voltage-clamp protocols and data acquisitions were performed using pCLAMP6 software (Axon Instruments, Inc.). Transfected cells were identified by the expression of GFP. The percentage of cells expressing GFP was about 40–60%, and Ca²⁺ channel currents were measured in about 60–80% of the GFP-expressing cells. Because the inactivation kinetics of Ca²⁺ channels are influenced by the current density, we used cells expressing Ca²⁺ channel current density between 10 and 100 pA/pF.

Single-channel Recording—Cell-attached single-channel recordings were performed with a high K⁺ bath solution to cancel membrane potential (in mM): 5 KCl, 112 potassium aspartate, 5 NaCl, 3 MgCl₂, 1 Mg-ATP, 2 EGTA, 10 glucose, 10 HEPES (pH 7.3, adjusted with KOH) at room temperature. The resistance of the heat-polished microelectrodes was between 2 and 9 MΩ when filled with the internal solution composed of (in mM): 110 BaCl₂, 10 HEPES (pH 7.4, adjusted with KOH) as measured using an oscilloscope (MT Corp., Seattle, WA), Igor Pro software, and Patch Analyst Pro software (MT Corp.).

Materials—Dilitiazem (generous gift from Tanabe Seiyaku) and verapamil (purchased from Nacalai Tesque, Kyoto, Japan) were dissolved in distilled water and stored at 4 °C as 1 mM stock solutions. Nitrrendipine (purchased from Funakoshi Seiyaku, Tokyo, Japan), S(-)Bay K 8644 (purchased from Sigma Chemical Co., St. Louis, MO), R(-)Bay K 8644 (purchased from Sigma), Bay Y 5959 (generous gift from Bayer AG, Wuppertal, Germany), (+)-iradipine (generous gift from Sandoz AG, Basel, Switzerland), and FPL-64716 (purchased from Sigma) were dissolved in ethanol and stored at -20 °C as 3 mM stock solutions. Drugs were dissolved in the external solution and applied by either perfusion (at 4 ml/min) or via concentration-clamp apparatus (Vibraspec, Inc., Philadelphia, PA) in the whole-cell patch-clamp recording. The concentration-clamp apparatus allowed us to exchange the external solution within 50 ms (17). In single-channel recording experiments, each drug was directly added to the bath solution to make up the final concentration.

Statistics—Data are expressed as means ± S.E. Statistical significance was assessed with the Student-Welch’s t test for simple comparisons. Differences at p < 0.05 were considered to be significant.

RESULTS

Expression and Electrophysiological Properties of Mutant a1C Subunits—We replaced the amino acids in III55-S6 linker and IVS5-S6 linker of rbcII, which were conserved in a1A, a2, a1C, a1D, a1B, and a1G, but not in TuCa1 or CyCa1, by corresponding amino acids of TuCa1 (S1115A, S1146A, and A1420S (Fig. 1)). Mutant a1C subunits were expressed in BHK6 cells that stably express rabbit β1A and α2δ subunits. Expression of these mutant Ca²⁺ channels in BHK6 cells were confirmed by checking its molecular mass by Western blotting (~220 kDa, data not shown).

First, we examined whether point mutations altered electrophysiological properties of L-type Ca²⁺ channels. Both activation and inactivation kinetics of Ca²⁺ channel currents of all mutants were almost identical to those of rbcII (data not shown). All of the mutant Ca²⁺ channels and rbcII were activated at threshold voltage around -40 mV and peaked at 0 mV, and the I-V curves of all channels coincided with each other (Fig. 2A). The half-activation voltage (V50) and slope were as follows: V50, slope: -15.9 ± 0.625 mV, -5.74 ± 0.381 (S1115A); -12.9 ± 0.577 mV, -7.54 ± 0.323 (S1146A); -13.2 ± 0.470 mV, -7.56 ± 0.263 (A1420S); -14.2 ± 0.484 mV, -6.65 ± 0.292 (rbcII), respectively (n = 8–14). Ca²⁺ channel current densities did not differ between mutated a1C subunits and rbcII (~60.8 ± 12.4 pA/pF (S1115A), ~40.1 ± 8.4
were measured with voltage protocol shown in B-a. Current traces are shown in the curves to two-exponential equation were as follows (differences in peak Ca$^{2+}$ are normalized by peak current amplitude. There were no significant differences in peak Ca$^{2+}$ channel current density between rbCII and mutant Ca$^{2+}$ channels. B, steady-state inactivation curves of Ca$^{2+}$ channel currents. Steady-state inactivation curves of S1115A (n = 6), S1146A (n = 12), A1420S (n = 8), and rbCII (n = 7) were measured with voltage protocol shown in B-a. Representative current traces are shown in B-b (rbCII). Pulses were applied every 30 s. C, restitution curve of Ca$^{2+}$ channel currents. Restitution curve of S1115A (n = 6), S1146A (n = 5), A1420S (n = 5), and rbCII (n = 7) were measured with voltage protocol shown in C-a. Representative current traces are shown in C-b (rbCII). Pulses were applied every 40 s. Normalized Ca$^{2+}$ channel current amplitudes (peak Ca$^{2+}$ channel currents of test pulse/peak Ca$^{2+}$ channel currents of conditioning pulse) are plotted in C-c. The horizontal axis indicates interval between conditioning pulse and test pulse. The error bars show S.E.

$pA/pF$ (S1146A), $-57.3 \pm 10.3$ $pA/pF$ (A1420S), $-47.8 \pm 6.9$ $pA/pF$ (rbCII), n = 8–14. There were no differences in steady-state inactivation curves ($V_{\text{half}}$ in mV): $-32.2 \pm 1.01$ (S1115A), $-34.3 \pm 0.970$ (S1146A), $-33.0 \pm 0.800$ (A1420S), $-30.0 \pm 0.810$ (rbCII) (n = 6–12; Fig. 2B, c). No differences were found in the restitution curves. Time constants estimated by fitting the curves to two-exponential equation were as follows ($\tau_{\text{fast}}$ and $\tau_{\text{slow}}$ in ms): (19.1 $\pm$ 5.6 and 157.2 $\pm$ 14.3 (S1115A), 14.5 $\pm$ 5.8 and 144.0 $\pm$ 17.2 (S1146A), 11.9 $\pm$ 4.1 and 127.1 $\pm$ 19.1 (A1420S), 12.8 $\pm$ 4.5 and 161.1 $\pm$ 17.2 (rbCII), respectively (n = 5–7; Fig. 2C, c). Therefore, S1115A, S1146A, and A1420S showed electrophysiological properties identical to those of rbCII.

**Sensitivity of Mutant Ca$^{2+}$ Channels to Various Ca$^{2+}$ Channel Antagonists**—First, we examined the effect of diltiazem. All mutants and rbCII were equivalently blocked by diltiazem (1 $\mu$m) at holding potentials of $-70$ and $-50$ mV without any differences in the voltage dependence of the block (Fig. 3A and B). We also compared the block of Ca$^{2+}$ channel currents by verapamil (1 $\mu$m, 10 $\mu$m). Ca$^{2+}$ channel currents of S1115A were blocked to the same degree as that of rbCII (Fig. 3C and D). On the other hand, sensitivity of S1115A to DHP antagonists, such as nitrrendipine, at a holding potential of $-70$ mV was significantly decreased compared with those of rbCII, S1146A, and A1420S (Fig. 4B). When holding potential was changed to $-50$ from $-70$ mV, nitrrendipine block of S1115A...
The IC\textsubscript{50} value for S1115A and S1115T are 57.5 and 25 M, respectively. In S1115A, the affinity for (+)-Bay K 8644 (1 M) (98.6 ± 3.45% of control) to the same degree as time control (92.9 ± 2.15% of control at 90 s, data not shown). Another DHP Ca\textsuperscript{2+} channel agonist, Bay Y 5959 (20), also did not enhance the Ca\textsuperscript{2+} channel current of S1115A, but rather slightly decreased it in a concentration-dependent manner (Fig. 5, C and D). These results demonstrate that S1115A is insensitive to DHP Ca\textsuperscript{2+} channel agonists. Thus we verified whether S1115A lacks the sensitivity to only DHP Ca\textsuperscript{2+} channel agonists or Ca\textsuperscript{2+} channel agonists generally. A benzoylpyrrole-type Ca\textsuperscript{2+} channel agonist, FPL-64176 binds to the Ca\textsuperscript{2+} channel at its binding site distinct from that of DHPs (21, 22). Ca\textsuperscript{2+} channel currents of S1115A were slighty enhanced by FPL-64176 (1 \mu M), unlike the results with S(-)-Bay K 8644 or Bay Y 5959. However, the enhancement of Ca\textsuperscript{2+} channel currents through S1115A were markedly smaller than that of rbCII (Fig. 5, E and F). Because Ser\textsuperscript{1115} is located only three amino acids away from Glu\textsuperscript{1118}, which is the determinant of Ca\textsuperscript{2+} selectivity of the \(\alpha_{1C}\) subunit, the sensitivity of S1115A for DHPs may be affected by charge carrier. Therefore, we tested whether the above results were affected when Ba\textsuperscript{2+} was used as a charge carrier in place of Ca\textsuperscript{2+}, and we obtained the same results as observed with Ca\textsuperscript{2+}, such as insensitivity to S(-)-Bay K 8644 (data not shown).

**Importance of the Hydroxyl Group of Ser\textsuperscript{1115}**—We replaced Ser\textsuperscript{1115} by other amino acid residues (Asp, Thr, and Val) to identify the functional group responsible for the action of Ca\textsuperscript{2+} channel agonists. Replacement by valine (S1115V) or aspartic acid (S1115D) abolished the effect of S(-)-Bay K 8644 (1 \mu M). In contrast, in S1115T, S(-)-Bay K 8644 produced slight but significant enhancement of Ca\textsuperscript{2+} channel currents (Fig. 6). The Ca\textsuperscript{2+} channel block produced by nitrendipine was also reduced in S1115T. The IC\textsubscript{50} value of S1115T was 1.9 \times 10^{-5} M, which was higher than that of rbCII by a factor of 25 but lower than that of S1115A by 2.3-fold (Fig. 4C). These results indicate that the hydroxyl group of Ser\textsuperscript{1115} forms part of the DHP binding pocket and plays a critical role in mediating the action of DHP Ca\textsuperscript{2+} channel agonists.

**Comparison of Unitary Ca\textsuperscript{2+} Channel Currents between rbCII and S1115A**—Single Ca\textsuperscript{2+} channel activity was recorded in cell-attached patch-clamp mode. In the presence of S(-)-Bay K 8644 or PFL-64176, the number of open events was largely increased in rbCII (NPo: N (the number of channels in the patch pipette) \* Po (open probability). 0.0099–0.138 by S(-)-Bay K 8644, 0.0244–0.828 by FPL-64176), but only slightly modified in S1115A (0.0296–0.0538, 0.00731–0.0132, by S(-)-Bay K 8644 and FPL-64176, respectively (Fig. 7A). Open-time histograms of rbCII and S1115A with and without S(-)-Bay K 8644 are shown in Fig. 7B. The mean open time of rbCII was also increased by S(-)-Bay K 8644 in rbCII but unchanged in S1115A (mean open time in ms: 1.60 ± 1.93 to 3.26 ± 3.23, 2.07 ± 3.46 to 2.24 ± 3.36, respectively). These results indicate that prolongation of Ca\textsuperscript{2+} channel opening by Ca\textsuperscript{2+} channel agonists is mostly absent in S1115A.

**DISCUSSION**

**Electrophysiological Properties of Mutant Ca\textsuperscript{2+} Channels**—DHP Ca\textsuperscript{2+} channel antagonists bind to Ca\textsuperscript{2+} channels in the inactivated state with high affinity (23). It is possible that the affinity of Ca\textsuperscript{2+} channel antagonists for Ca\textsuperscript{2+} channels is influenced by their inactivation kinetics. However, we did not find any differences in the electrophysiological properties between rbCII and mutant \(\alpha_{1C}\) subunits, indicating that the mutation introduced into the \(\alpha_{1C}\) subunit did not affect the inactivation kinetics (Fig. 2). Therefore, any differences in the responsiveness to DHPs between rbCII and mutant Ca\textsuperscript{2+} channels should result from the conformational change of the DHP binding pocket or the gating moiety linked to the DHP-binding site.
Pharmaco-logical Properties of S1115A—IC50 values of the Ca2+ channel blocker produced by nitrendipine was higher in S1115A by 57.5-fold than in rbCII. Another DHP Ca2+ channel antagonist, R(+)-Bay K 8644, also produced significantly smaller block in S1115A than in rbCII (Fig. 4). The mutation in IIIS6 (Y1152A) has been shown to reduce the affinity of (+)-isradipine by 25-fold (12). In the present study, the affinity of (+)-isradipine for S1115A was also lower than that of rbCII by 24-fold. These results indicate that the replacement of Ser1115 by Ala reduced the affinity for DHP antagonists. In contrast, the Ca2+ channel currents of S1115A were rather slightly inhibited by another DHP Ca2+ channel antagonist, FPL-64176 at 1 and 10 μM, respectively. 

Therefore, Ser1115 in IIIS6-S6 linker appears to play a critical role in the binding pocket specific to DHPs.

Unexpectedly, Ca2+ channel currents of S1115A were insensitive to DHP Ca2+ channel agonists and rather slightly decreased by S(−)-Bay K 8644 (Fig. 5, A and B) in a way similar to that of time control (data not shown). R(−)-Bay K 8644, a DHP Ca2+ channel antagonist, and S(−)-Bay K 8644, a DHP Ca2+ channel agonist, are enantiomers. However, effects of both chemical compounds were not linearly altered in S1115A: antagonistic effects of R(−)-Bay K 8644 were reduced, but agonistic effects of S(−)-Bay K 8644 were completely eliminated. Even higher concentration (3 μM) of S(−)-Bay K 8644, a concentration more than 300 times higher than EC50 value of S(−)-Bay K 8644 (−10 nM), did not enhance Ca2+ channel currents of S1115A (data not shown). Thus the impairment of the action of both stereoisomers of DHPs were not simply explained by a decrease of affinity. Therefore, Ser1115 appears to serve as an important site for agonistic action of DHP Ca2+ channel agonists. Indeed, Ca2+ channel currents of S1115A were also rather slightly inhibited by another DHP Ca2+ channel agonist, Bay Y 5959, regardless of concentration (Fig. 5, C and D). In contrast, the benzoylpyrrole (non-DHP) Ca2+ channel agonist, FPL-64176, slightly enhanced Ca2+ channel currents of S1115A, but the effect was significantly smaller than that of rbCII (Fig. 5, E and F). Considering that the bindings of S(−)-Bay K 8644 and FPL-64176 to the Ca2+ channel do not compete with each other, i.e. that the binding sites for both compounds are distinct, the two compounds may share the azem and verapamil in the same way as that of rbCII (Fig. 3).
role in the action of Ca$^{2+}$ it was unchanged in S1115A (2.07 ± 0.05 ms vs. 1.23 ± 0.03 ms in rbCII) (data not shown). The single-channel study clearly showed that, in S1115A, Ca$^{2+}$ channel agonists failed to prolong Ca$^{2+}$ channel opening. However, unexpectedly, the open probability was slightly enhanced by S(-)-Bay K 8644 (Fig. 7A), although the whole-cell Ca$^{2+}$ channel current of S1115A was not increased at all (Fig. 5A). These somewhat contradictory results may be explained as follows: 1) in whole-cell recording, natural run-down of the Ca$^{2+}$ channel current may have masked the slight enhancement of S1115A by S(-)-Bay K 8644 and resulted in the slight decrease of the current, because S(-)-Bay K 8644 reduced the Ca$^{2+}$ channel current to a degree similar to that of time control; 2) the difference of charge carrier (Ca$^{2+}$ and Ba$^{2+}$) may influence the effects of S(-)-Bay K 8644, because Ser$^{1115}$ is located near a Ca$^{2+}$-selective filter (Glu1118). However, the latter possibility is unlikely, because effects of S(-)-Bay K 8644 on whole-cell Ca$^{2+}$ channel currents were the same in rbCII and S1115A whether the charge carrier was Ca$^{2+}$ (2 mM) or Ba$^{2+}$ (2 mM) (data not shown).

Importance of the Hydroxyl Group of Ser$^{1115}$—We replaced

common site, such as Ser$^{1115}$, for producing Ca$^{2+}$ channel agonist effects. These results indicate that Ser$^{1115}$ plays a critical role in the action of Ca$^{2+}$ channel agonists.

Binding of Ca$^{2+}$ to the Ca$^{2+}$ channel pore may contribute to stabilize high affinity DHP binding (5, 24). However, the direct interaction between the Ca$^{2+}$-binding site of the Ca$^{2+}$ channel pore with DHPs has not been shown. The critical role of Ser$^{1115}$ in DHP binding strongly supports this idea, because Ser$^{1115}$ is located at three amino acids away from the key Glu$^{1118}$ of the channel pore (25) (Fig. 8A). In addition, our findings are consistent with the previous studies: 1) DHP agonists and antagonists gain access to their binding sites from the extracellular surface of the cell membrane (26–29), 2) the DHP-binding site resides 11–14 Å from the extracellular side of the channel, 2) the DHP-binding site resides 11–14 Å from the extracellular surface of the cell membrane (26–29), 3) photoreactive DHPs specifically label the connecting loop between IIIS5 and IIIS6 (30–33), 4) in analysis of chimeric Ca$^{2+}$ channels, IIIS5-S6 linker is necessary for transferring DHP binding, which were basically determined by comparison between the sequences of the DHP-insensitive $\alpha_{1A}$ subunit and the DHP-sensitive $\alpha_{1C}$ subunit. However, the alanine-scanning mutagenesis performed in the whole area of the transmembrane region IIIS6 and IVS6 showed that the DHP binding pocket includes amino acids conserved in both $\alpha_{1A}$ and $\alpha_{1C}$ subunits. For this reason, the amino acids highly conserved in both $\alpha_{1A}$ and $\alpha_{1C}$ subunits may also serve as critical sites for DHP binding. Our finding of Ser$^{1115}$ in IIIS5-S6 linker as a novel determinant of DHP binding is the first step toward understanding the molecular mechanism underlying the conformational change of the pore region involved in the modulation of Ca$^{2+}$ channel gating by DHPs. Involvement of other domains such as motifs IIIS6-S6 and IIIS5-S6 are under investigation.

The single-channel study clearly showed that, in S1115A, Ca$^{2+}$ channel agonists (or DHP agonist) failed to prolong Ca$^{2+}$ channel opening. However, unexpectedly, the open probability was not increased at all (Fig. 5A). These somewhat contradictory results may be explained as follows: 1) in whole-cell recording, natural run-down of the Ca$^{2+}$ channel current may have masked the slight enhancement of S1115A by S(-)-Bay K 8644 and resulted in the slight decrease of the current, because S(-)-Bay K 8644 reduced the Ca$^{2+}$ channel current to a degree similar to that of time control; 2) the difference of charge carrier (Ca$^{2+}$ and Ba$^{2+}$) may influence the effects of S(-)-Bay K 8644, because Ser$^{1115}$ is located near a Ca$^{2+}$-selective filter (Glu1118). However, the latter possibility is unlikely, because effects of S(-)-Bay K 8644 on whole-cell Ca$^{2+}$ channel currents were the same in rbCII and S1115A whether the charge carrier was Ca$^{2+}$ (2 mM) or Ba$^{2+}$ (2 mM) (data not shown).

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Ser\(^{1115}\) by other amino acid residues (Asp, Thr, Val) to identify the important functional group of this serine. Only S1115T was slightly increased by S-(−)-Bay K 8644. The binding affinity of S1115T for DHP antagonist was significantly lower than that of rbCII but higher than that of S1115A. These results indicate that the hydroxyl group at Ser\(^{1115}\) is important for DHP binding and the action of Ca\(^{2+}\) channel agonists. The steric hindrance by the methyl group at β-carbon of threonine may be the reason why effects of DHPs were significantly reduced in S1115T.

The conformational dynamics of IIIS5-S6 linker and its role in the gating mechanism have not been clarified yet. When Gln\(^{1043}\) in IIIS5 (10) and Met\(^{1161}\) in IIIS6 (35) were replaced, agonistic effects of DHPs were selectively abolished, while antagonistic effects remained. Therefore, these two amino acids may be responsible for the conformational change of IIIS5-S6 caused by DHP Ca\(^{2+}\) agonists. Based on our finding that Ser\(^{1115}\) contributes to DHP binding and plays a critical role in producing the action of Ca\(^{2+}\) channel agonists, Ser\(^{1115}\) may be involved, in cooperation with Gln\(^{1043}\) and Met\(^{1161}\), in the conformational change of IIIS5-S6 linker upon binding of DHP Ca\(^{2+}\) channel agonists. Considering that electrophysiological properties of S1115A were identical to those of rbCII, Ser\(^{1115}\) appears to be a critical amino acid that links the binding of DHP agonists and the modulation of Ca\(^{2+}\) channel gating, which is not a result of modulation of Ca\(^{2+}\) channel activation (36, 37) nor inactivation (34, 38) but rather may be the result of direct stabilization of the Ca\(^{2+}\) channel pore in the open state. Our finding further implies that the conformational change of IIIS5-S6 linker may be a critical step of Ca\(^{2+}\) channel gating and its modulation by Ca\(^{2+}\) channel antagonists or agonists. Therefore, we propose a model that DHPs interact with the hydroxyl group of Ser\(^{1115}\) and, especially upon the binding of Ca\(^{2+}\) channel agonists, such interaction stabilizes the Ca\(^{2+}\) channel in the open state (Fig. 8B).

Summary—We identified a novel critical site (Ser\(^{1115}\) in the IIIS5-S6 loop of the L-type Ca\(^{2+}\) channel α\(_{1C}\) subunit) for DHP binding. Ser\(^{1115}\) turned out to be a critical determinant of action of DHP agonists.

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