Transfer of the High Affinity Dihydropyridine Sensitivity from L-Type To Non-L-Type Calcium Channel

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SUMMARY

To elucidate the mechanism underlying the interaction between the L-type Ca^{2+} channel and the dihydropyridines (DHPs), contribution of the repeat III was studied by constructing chimeras between the DHP-sensitive $\alpha 1C$ and DHP-insensitive $\alpha 1E$ subunits. The chimeras were transiently expressed in human embryonic kidney 293 cells and the whole-cell Ba^{2+} current (I_{Ba}) was recorded. Mutating Thr1061 to Tyr in IIIS5 of the $\alpha 1C$ sequence completely abolished the inhibition and stimulation of I_{Ba} by the antagonist (+)-isradipine and agonist (−)-Bay K 8644, whereas mutating Gln1065 to Met in IIIS5 decreased the affinity for isradipine 100-fold without affecting the stimulating effect of Bay K 8644. The conserved amino acid residue Tyr1174 in IIIS6 of the $\alpha 1C$ subunit was necessary for the high affinity DHP block. The DHP-dependent block and stimulation of I_{Ba} were transferred to the $\alpha 1E$ channel by the mutation of two amino acid residues in IIIS5 (Y1295T, M1299Q), three residues in IIIS6 (F1406I, F1409I, V1414M) and three residues in IVS6 (I1706Y, F1707M, L1714I). The mutated $\alpha 1E$ channel was stimulated 2.8-fold by 1 μM Bay K 8644 and blocked by isradipine with an IC_{50} value of 60 nM. These results show that mutation of Thr1061 in the $\alpha 1C$ sequence results in a DHP-insensitive L-type channel and that transfer of the high affinity DHP sensitivity requires mutation of eight amino acid residues in the $\alpha 1E$ sequence.

The voltage-gated Ca^{2+} channels mediate various cell functions, including excitation-contraction coupling, signal transduction, and neurotransmitter release. To date, six genes have been identified for the $\alpha 1$ subunit, which is the principal subunit of the Ca^{2+} channel (for review, see Refs. 1 and 2). Electrophysiological and pharmacological techniques have been used to correlate the naturally expressed channels with the identified genes (3). These studies showed that the $\alpha 1C$, $\alpha 1D$, and $\alpha 1S$ genes code for DHP-sensitive Ca^{2+} channels, whereas the channels encoded by the $\alpha 1A$, $\alpha 1B$, and $\alpha 1E$ genes are not affected by the DHPs (for review, see Ref. 4). The $\alpha 1C$ subunit along with the β and $\alpha 2/\delta$ subunits compose the L-type Ca^{2+} channel present in tissues such as the cardiac muscle, smooth muscle, and neurons. One of the clinically important features of the L-type Ca^{2+} channels is their sensitivity to the DHPs. In spite of their importance, the molecular basis of their action on the Ca^{2+} channel is still not fully understood.

Photoaffinity-labeling studies using the skeletal muscle calcium channel proposed several sites for the DHP channel interaction, including the pore region and the last transmembrane segment (S6) in repeats III and IV (5–8). Site directed mutagenesis of the $\alpha 1C$ and the $\alpha 1S$ subunit indicated that the putative transmembrane segments S5 and S6 in repeat III and the three amino acids Tyr1485, Met1486, and Ile1493 in IVS6 contribute to the high affinity interaction of the DHPs with the $\alpha 1$ subunits (9–11). Transfer of these regions to the DHP-insensitive $\alpha 1A$ subunit created a mutant channel, which was inhibited by moderate concentrations of the DHP antagonist isradipine (12). Point mutations in the IIIS6 segment indicated that the transfer of the DHP sensitivity to the $\alpha 1A$ subunit required, in particular, Thr1061 and Gln1065 present in IIIS5 of the $\alpha 1C$ subunit (13).

So far, the minimal number of amino acid residues required for the transfer of the DHP sensitivity is unclear. We used the following strategy to identify these residues. First, we examined the contribution of amino acid residues in the segments IIIS5, IIIS6, and IVS6 to the DHP sensitivity of the L-type $\alpha 1C$ channel. Then we transferred the identified amino acid residues to the DHP-insensitive $\alpha 1E$ channel to create a non–L-type, DHP-sensitive channel. It is shown that the single point mutation of Thr1061 to Tyr (i.e., replacing an alcohol hydroxyl group with a phenol hydroxyl group), abolishes inhibition and stimulation of the $\alpha 1C$ subunit-mediated barium current (I_{Ba}) by DHPs. DHP-dependent inhibition and stimulation of the $\alpha 1E$-mediated current requires the replacement of eight amino acid residues and the presence of Tyr1405 in IIIS6.

ABBREVIATIONS: DHP, dihydropyridine; I_{Ba}, barium current; I-V, current-voltage; HEK, human embryonic kidney; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid.

Materials and Methods

Chemicals. The restriction enzymes were purchased from New England Biolabs (Beverly, MA), Boehringer-Mannheim Biochemicals (Mannheim, Germany) or Amersham (Buckinghamshire, UK). Lipofectamine was purchased from Gibco/BRL (Gaithersburg, MD). The Ca^{2+} channel blockers were obtained from sources mentioned previously (11).

Construction of chimeras. The EC chimeras were constructed by subcloning the sequence between *Bbr*PI (nt 3126) and *Sac*II (nt 4101) sites of the rabbit α1C-b cDNA (14) into the pUC18 vector (Pharmacia Biotech, Uppsala, Sweden). The constructs were then cloned into pcDNA3 vector (InVitrogen, Carlsbad, CA) to form the full-length construct. Two silent mutations were introduced to create a *Sal*I (nt 3411) and a *Not*I (nt 3664) restriction site. Part of the insert encoding the region between IIIS5 and IIIS6 was replaced with the corresponding sequence of the human brain α1E cDNA (from nt 3870 to nt 4263; numbers according to Ref. 15) by using the standard polymerase chain reaction overlap extension technique. The same method was used to construct the "rEC" chimeras. These "reverse" chimeras were constructed by subcloning the sequence between *Sse*8387I (nt 3635) and *Sfi*I (nt 5786) sites of the α1E cDNA (15), and two silent mutations were introduced to create a *Bgl*II (nt 4969) and an *Hpa*I (nt 5300) restriction site. The sequence of each chimera was confirmed by sequence analysis. The following amino acid sequences of α1C were replaced with the corresponding sequences of α1E (numbers according to α1C sequence): EC1 (1067–1191), EC2 (1167–1191), EC4 (1067–1076). The mutations of EC5–18 and rEC2–10 are indicated in Figs. 2, 5, and 6. The numbers of the amino acid residues for EC5–18 and rEC2–10 are those of the α1C-b subunit (14) and α1E subunit (15), respectively.

Expression of Ca^{2+} channel and electrophysiological recording. The methods for the channel expression and current recordings are essentially the same as those described previously (11). HEK 293 cells were transiently transfected with the plasmids encoding the wild-type or chimeric α_1 subunits together with the plasmids encoding β2a and α2/δ subunits by lipofection at a DNA mass ratio of 1:1:1. The patch clamp experiments were performed by using EPC-9 amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany). The whole-cell I_{Ba} was induced by a 40-msec square pulse at 0.2 Hz either from a holding potential of -80 mV to $+10$ or $+20$ mV (wild-type α1C and the "EC" chimeras), or from -100 mV to 0 mV or $+10$ mV (wild-type α1E and the "rEC" chimeras), according to the peak I-V relation of each cell. The extracellular bath solution contained: 82 mM NaCl, 20 mM tetraethyl ammonium chloride, 30 mM $BaCl_2$, 5.4 mM CsCl, 1 mM $MgCl_2$, 0.1 mM EGTA, 5 mM HEPES, 10 mM glucose, pH 7.4 (adjusted by NaOH). The intracellular pipette solution contained: 102 mM CsCl, 10 mM tetraethyl ammonium chloride, 10 mM EGTA, 1 mM $MgCl_2$, 3 mM Na_2ATP, 5 mM HEPES, pH 7.4 (adjusted by CsOH). The input capacitances of the cells were between 15 and 60 pF. All experiments were performed at room temperature.

Data analysis. The current recordings were filtered at 2 kHz and sampled at 10 kHz unless otherwise stated. Data were stored on-line and analyzed using either the standard program package provided by HEKA Elektronik or Origin (Microcal Software, Northampton, MA). The effects of the drugs were evaluated by measuring the peak amplitude of I_{Ba}. Statistical analysis was performed using Student's t test and values of $p < 0.05$ were considered to be significant. Statistical data are given as the mean $\pm$ standard error.

Results and Discussion

Contribution of the repeat III to the DHP sensitivity of the L-type Ca^{2+} channel. Previously, it has been suggested that the region between IIIS5 and IIIS6 contributes significantly to the high affinity interaction of the DHP isradipine with the α1C subunit (5, 6, 8–10, 12). To examine whether this region of the α1C subunit is necessary for the interaction with isradipine, the sequence of this region was substituted with that of the DHP-insensitive α1E subunit (Fig. 1A). The resulting chimera EC1 was not inhibited significantly by 3 μM (+)-isradipine, whereas I_{Ba} of the wild-type α1C channel was completely suppressed at this drug concentration (Fig. 1B). This drug concentration is 250-fold higher than the IC_{50} value (12.2 $\pm$ 0.7 nM) for the wild-type channel. Refinement of this relative large sequence region showed that the replacement of the IIIS6 segment (EC2) shifted the IC_{50} value to a 4.5-fold higher value (55.5 $\pm$ 4.6 nM; Fig. 1B), which indicates that this segment contributes to the interaction of isradipine with the channel (see also experiments described below). In contrast, the replacement of the IIIS5 segment (EC4) resulted again in the loss of the DHP block (Fig. 1B) suggesting that amino acid residues in the IIIS5 segment are essential for a high affinity block of the α1C channel.

Thr1061 of IIIS5 is required for the DHP block. The transmembrane segment IIIS5 of the α1 subunits differs in seven amino acid residues between the DHP-sensitive and -insensitive Ca^{2+} channels (Fig. 1C). Replacement of one to five of these seven amino acid residues suggested that mutation of Gln1065 to Met (EC17) decreased the antagonistic effect of 3 μM isradipine from 100% to 60% (Fig. 2). On the other hand, the isradipine block was completely wiped out by the replacement of Thr1061 with Tyr (EC14), which shows that Thr1061 is essential for the high affinity DHP block of the α1C channel (Fig. 2). The point mutation T1061Y is remarkable, because it only replaces an alcohol hydroxyl group with a phenol hydroxyl group. The specificity of the mutations T1061Y and Q1065M for the DHPs was further
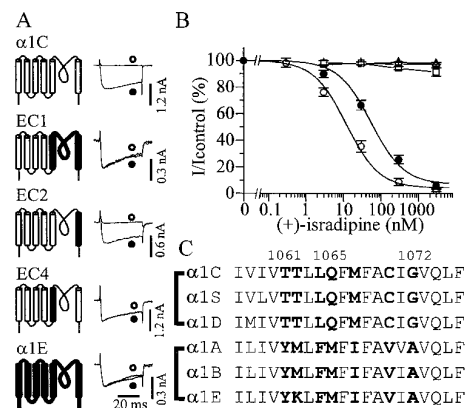

Fig. 1. Contribution of repeat III to the DHP blocking effect. A, Schematic structure (*left*) and whole-cell I_{Ba} (*right*) of wild-type and chimeric Ca^{2+} channels. For the schema, only the constructs of the repeat III for the wild-type or chimeric α1 subunits are shown. Current traces were recorded before (●) and during (○) the superfusion of 3 μM (+)-isradipine. B, Dose-response curves of (+)-isradipine for the inhibition of peak I_{Ba} recorded in wild-type α1C (○) and α1E (▲), and chimeras EC1 (□), EC2 (●), and EC4 (△). *Points,* mean $\pm$ standard error from 4–12 cells. For the wild-type α1C and EC2 channels, *smooth lines* were drawn by fitting the *data points* with the Hill equation, which gave the IC_{50} values of 12.2 $\pm$ 0.7 nM (11 experiments) and 55.5 $\pm$ 4.7 nM (six experiments) for the wild-type α1C and EC2, respectively. Note that the *data points* for the wild-type α1E and EC4 are nearly identical. C, Amino acid sequence alignment of the IIIS5 for the six Ca^{2+} channel α1 subunits. *Bold letters,* amino acid residues that are different between the DHP-sensitive (α1C, α1S, and α1D) and DHP-insensitive (α1A, α1B, and α1E) Ca^{2+} channels. *Numbers on the top,* amino acid residues refer to the α1C sequence (14).

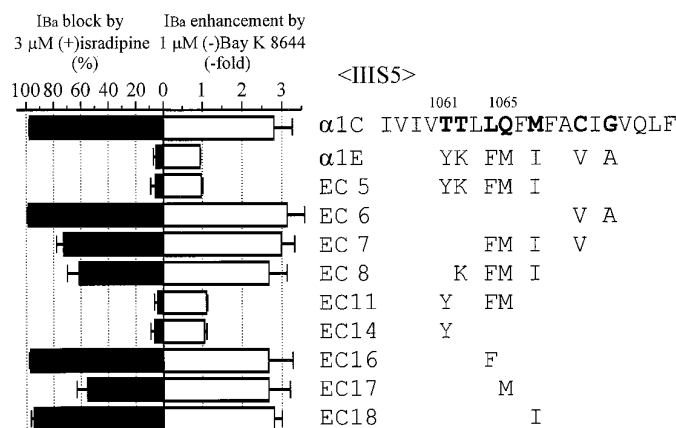Fig. 2. Thr1061 in IIIS5 is critical for the DHP sensitivity of α1C channel. *Left*, response of I_{Ba} to 3 μM (+)-isradipine and 1 μM (−)-Bay K 8644 recorded from the wild-type or chimeric Ca^{2+} channels. Values are the mean ± standard error from 4–11 cells. *Right*, amino acid alignment of the wild-type and chimeric Ca^{2+} channels. For the wild-type α1E and the chimeras, only the amino acid residues that are different from those of the wild-type α1C sequence are presented.
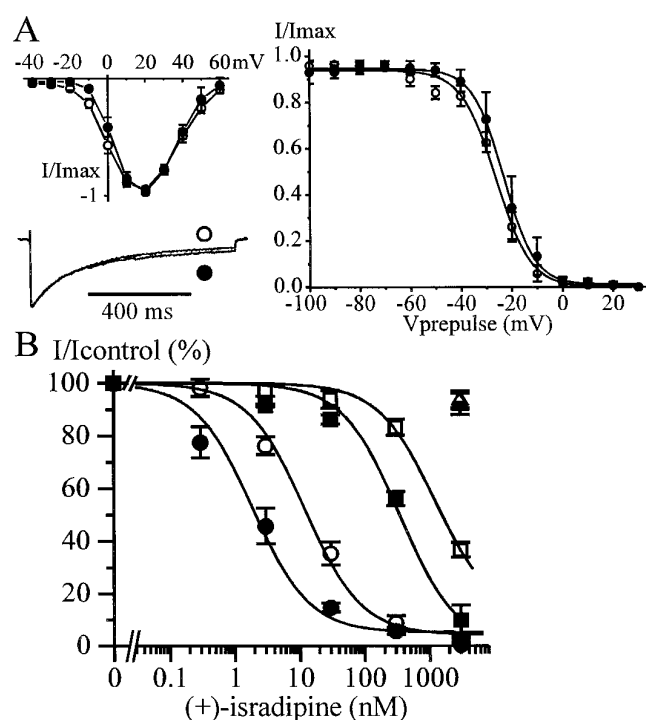

Fig. 3. Electrophysiological and pharmacological properties of the chimeras. A, the basal electrophysiological properties of the wild-type α1C (○) and EC14 (●) channel. *Upper left*, I-V relation of the wild-type α1C (12 experiments) and EC14 (9 experiments). *Lower left*, comparison of the time course of the current inactivation recorded with 800-msec square pulses from −80 mV to +20 mV and sampled at 1 Hz. The current decay was fitted with two exponentials, with the time constants of 86.9 ± 8.9 msec and 336.7 ± 34.6 msec for the wild-type α1C (10 experiments) and 85.9 ± 15.3 msec and 330.2 ± 29.1 msec for EC14 (11 experiments). The relative amplitudes of the fast component were 43.7 ± 7.9% and 38.6 ± 5.1% for the wild-type α1C and EC14, respectively. These values were not significantly different between the wild-type α1C and EC14 channels. The currents are scaled to the same peak amplitude for easier comparison. The actual peak current amplitudes were −1.8 nA and −2.6 nA for the wild-type α1C and EC14 channels, respectively. *Right*, steady state inactivation was obtained by measuring the peak current amplitude of a 100-msec test pulse to +10 mV or +20 mV (voltage showing the peak I-V relation), which was applied after a 5-sec prepulse to various potentials and a 10-msec return to the holding potential of −80 mV every 25 sec. Data were sampled at 2 kHz. *Smooth lines,* obtained by fitting the *data points* with the Boltzmann equation. The half-maximum inactivation potentials were −26.1 ± 1.4 mV and −22.9 ± 3.8 mV and the slope factors were 6.9 ± 1.1 mV and 4.4 ± 0.8 mV for the wild-type α1C (five experiments) and EC14 (five experiments) channels, respectively, which were not significantly different. B, Dose-response curves for (+)-isradipine on the wild-type α1C (○, ●) and EC17 (□, ■) and effect of 3 μM (+)-isradipine on EC14 (△, ▲) channel. I_{Ba} was recorded either at the holding potential of −80 mV (○, □, △) or −40 mV (●, ■, ▲). *Smooth lines,* fitted with the Hill equation. IC_{50} values at −80 mV were 12.2 ± 1.9 nM (11 experiments) and 1.22 ± 0.16 μM (six experiments) and those at −40 mV were 1.88 ± 0.22 nM (five experiments) and 0.28 ± 0.09 μM (four experiments), for the wild-type α1C and EC17 channels, respectively. In EC14, 3 μM isradipine decreased I_{Ba} to 93.2 ± 2.9% (seven experiments) and 91.2 ± 4.5% (four experiments) of the control when recorded at the holding potential of −80 mV and −40 mV, respectively.

analyzed by determining whether or not the IC_{50} values for the phenylalkylamine (−)-devapamil and the new Ca^{2+} channel blocker mibefradil were shifted by these mutations in chimera EC5. The phenylalkylamine gallopamil is reported to block the α1E channel current only partially at a high concentration of 10 μM (15), whereas the IC_{50} value for mibefradil is reported to be severalfold smaller for the α1E channel than the α1C channel (16). If the mutations in EC5 include the amino acid residues responsible for the effects of the two non-DHP blockers, the IC_{50} value is expected to increase for devapamil and/or decrease for mibefradil. On the other hand, the chimera with the mutation T1061Y may not be blocked by these two compounds, which suggests that the mutation disrupted nonspecifically the interaction between the channel and these Ca^{2+} channel blockers. The IC_{50} values for the two drugs were not significantly different between the wild-type α1C and EC5 channel, ruling out these two possibilities. The IC_{50} values for devapamil were 3.05 ± 0.56 μM (five experiments) and 3.12 ± 1.10 μM (four experiments) and those for mibefradil were 1.19 ± 0.03 μM (five experiments) and 1.14 ± 0.02 μM (five experiments) for the wild-type α1C and EC5 channels, respectively. These results indicate that the point mutations specifically disrupt the interaction with the DHPs and do not affect the block by other Ca^{2+} channel blockers.

The T1061Y mutation (EC14) had no significant effect on the basic electrophysiological parameters of the channel (Fig. 3A). The I-V relation, the time course of the channel inactivation, the steady state inactivation did not differ significantly between the wild-type α1C and EC14 channels. These findings did not rule out that the T1061Y mutation affected the voltage-dependent interaction of the channel with isradipine. The affinity of the L-type Ca^{2+} channel for the DHPs increases by depolarizing the holding potential, because such a shift of the holding potential increases the number of channels entering the inactivated state (17, 18). As expected, the shift of the holding potential from −80 mV to −40 mV decreased the IC_{50} values for the wild-type α1C channel from 12.2 ± 1.9 nM to 1.88 ± 0.22 nM and those for the Q1065M (EC17) channel from 1.22 ± 0.16 μM to 0.28 ±

0.09 μM (Fig. 3B). However, the same shift in the holding potential did not increase the affinity of the T1061Y (EC14) channel for isradipine (Fig. 3B). These findings show that the loss of the DHP sensitivity of chimera EC14 was caused by the disruption of the drug interaction site itself and not by

the modification of the basal electrophysiological properties of the channel.

Thr1061 of IIIS5 is required for the DHP stimulation. Previous mutations of the α1C channel in the IVS6 segment showed that mutation of three amino acids was necessary to decrease the inhibitory effect of isradipine, whereas mutating only two of the three amino acids was sufficient to abolish the stimulatory effect of $(-)$-Bay K 8644 (11). As shown in Fig. 2, Bay K 8644-induced stimulation of I_{Ba} was abolished by the point mutation T1061Y (EC14), further strengthening the above conclusion that this mutation disrupts the DHP interaction site. The differential effects of Bay K 8644 on the single point mutants T1061Y (EC14) and Q1065 M (EC17) were investigated in detail (Fig. 4). In the wild-type α1C and EC17 channel, the agonist slowed the deactivation of the tail current and shifted the peak I-V relation to the negative potential by 10 to 20 mV, along with increasing the current amplitude (upper and lower panel in Fig. 4). In contrast, the mutation T1061Y in EC14 completely abolished the stimulating effect and kinetic changes induced by Bay K 8644 (Fig. 4, middle panel). These results clearly demonstrate that Thr1061, but not Gln1065, is essential for the stimulatory effect of Bay K 8644 in the wild-type α1C channel.

Introduction of the DHP sensitivity to the non–L-type Ca^{2+} channel. By replacing the amino acid residues in the α1C subunit with those in the α1E subunit, we identified the two amino acid residues in IIIS5, Thr1061 and Gln1065, that are important for the DHP sensitivity of the wild-type α1C channel. Previous studies using similar methods indicated that three amino acid residues in the IVS6 (Tyr1485, Met1486, and Ile1493) also contribute to the DHP sensitivity of the L-type Ca^{2+} channels (10, 11). The importance of these amino acid residues for the DHP interaction site was tested

by introducing these amino acid residues into the sequence of the α1E subunit, creating reverse chimeras. The combined reverse mutations of I1706Y, F1707M, and L1714I in IVS6 (rEC2) or the single reverse mutation of either Y1295T (rEC4) or M1299Q (rEC5) in IIIS5 could not introduce the DHP block or stimulation to the α1E channel (Fig. 5). Reverse mutation Y1295T in combination with either M1299Q (rEC6) or the three amino acid residues in IVS6 (rEC7) was required for the significant isradipine block of the α1E channel, although 3 μM isradipine blocked I_{Ba} only by 35.2 $\pm$ 2.9% (five experiments) and 21.2 $\pm$ 1.0% (five experiments) in rEC6 and rEC7, respectively. Without Y1295T, the reverse mutations of the remaining four amino acid residues were not sufficient for the DHP sensitivity (see rEC8). This finding is consistent with the results shown in Fig. 2 and the previous reports (13, 19), supporting the critical role of Thr1061 of the α1C subunit for the interaction of the channel with the DHPs. The extent of the isradipine block increased when all the five reverse mutations, (Y1295T, M1299Q, I1706Y, F1707M, and L1714I) were combined (rEC9). In rEC9, 3 μM isradipine blocked I_{Ba} by 79.6 $\pm$ 1.3% (six experiments) and the dose-response curve yielded an IC_{50} value of 0.36 $\pm$ 0.03 μM (six experiments; Fig. 7B).

The responsiveness of the reverse chimeras for the DHP agonist Bay K 8644 was different from that seen for the antagonist isradipine. In rEC9, Bay K 8644 increased I_{Ba} 1.8 $\pm$ 0.2-fold (six experiments), whereas the stimulatory effect of the drug was not observed in the other reverse chimeras (Fig. 5). This stimulation required the double mutation Y1295T and M1299Q in IIIS5. The single mutations of Y1295T (rEC7) or M1299Q (rEC8) were insufficient to support agonist stimulation of the α1E chimera. In addition to the mutations in IIIS5, the mutations in IVS6 were necessary as exemplified in chimera rEC6. In this chimera, Bay K 8644 decreased I_{Ba} by 22.7 $\pm$ 2.9% (five experiments), which was consistent with our previous finding (11) and probably reflects the dual agonist and antagonistic nature of the enantiomer (20, 21). The replacement of Gln1065 in the α1C sequence by Met (EC17 in Fig. 2) did not significantly affect the sensitivity to Bay K 8644, whereas the absence of this amino acid in the α1E sequence (rEC8 in Fig. 5) resulted in the loss of Bay K 8644 stimulation. This difference could be caused by the presence of other unidentified amino acid res-
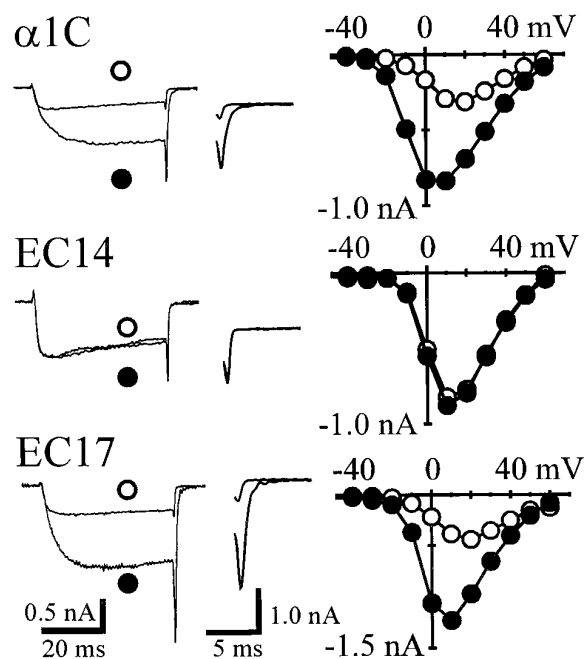

Fig. 4. The stimulatory effect of Bay K 8644 is abolished in chimera EC14. Current traces recorded at the potentials of the peak I-V relations (*left*) and the I-V relations obtained from the same cells (*right*). In each cell, I_{Ba} was first recorded under the control condition ($\bigcirc$) and then superfused with 1 μM Bay K 8644 ($\bullet$). The traces of the tail currents are also shown in an expanded time scale.
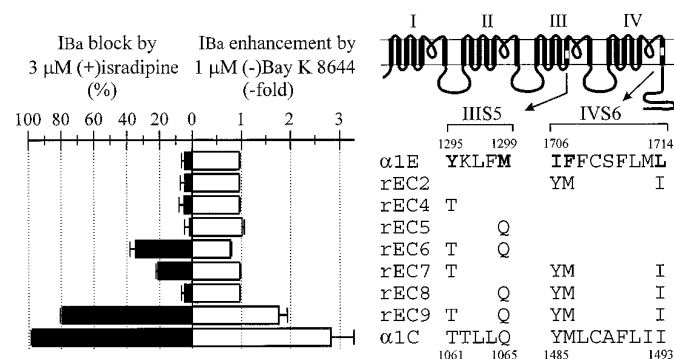

Fig. 5. Transfer of the DHP sensitivity to the α1E channel. *Left*, response of I_{Ba} to 3 μM (+)-isradipine and 1 μM (−)-Bay K 8644 recorded from the wild-type or chimeric Ca^{2+} channels. Values are the mean $\pm$ standard error from 5–11 cells. *Right*, amino acid alignment of the wild-type and chimeric Ca^{2+} channels. For the chimeras, only the mutated amino acid residues are indicated.

idues in the α1C sequence that allow stimulation of the channel in the absence of Gln1065.

Contribution of amino acid residues in IIIS6 to the DHP sensitivity. Although mutating five amino acid residues of IIIS5 and IVS6 in α1E (rEC9) remarkably increased the DHP sensitivity with the IC$_{50}$ value of 0.36 μM, the sensitivity was still not as high as in the wild-type α1C channel, which showed an IC$_{50}$ value of 12 nM. The substitution of IIIS6 of the α1C channel with that of the α1E channel (EC2) resulted in a 4.5-fold decrease in the isradipine sensitivity (Fig. 1), which indicates that the amino acid residues in this transmembrane segment are necessary for the DHP interaction. It has been suggested that three amino acid residues in IIIS6 (Ile1175, Ile1178, and Met1183) contribute to the DHP sensitivity (10, 22). We introduced three mutations, F1406I, F1409I, and V1414M, to the reverse chimera rEC9 yielding the reverse chimera rEC10 (Fig. 6A). These additional mutations improved considerably the DHP sensitivity of the α1E channel. I$_{Ba}$ of the rEC10 channel was almost completely blocked by 3 μM isradipine, and the IC$_{50}$ value was shifted 6-fold to 57.5 nM compared with the rEC9 channel (Fig. 6B). Bay K 8644 stimulated I$_{Ba}$ of the rEC10

channel 2.8 ± 0.3-fold (five experiments) and introduced the kinetic changes [i.e., negative shift of the peak I-V relation and the slowing of the tail current in two-thirds of the cells (Fig. 6C)]. I$_{Ba}$ of the remaining one-third of the cells was stimulated 2–3-fold by Bay K 8644 without obvious changes in the kinetics.

The results presented thus far have focused on the amino acid residues that are different between the DHP-sensitive α1C subunit and DHP-insensitive α1E subunit. This approach neglects the potential necessity of the conserved residues for the DHP interaction with the channels. In a radioligand binding study, it was reported that mutating Tyr in IIIS6 of the α1S subunit to Ala resulted in a marked decrease in the DHP sensitivity of the channel (10). The corresponding amino acid residues, Tyr1174 for the α1C and Tyr1405 for the α1E (Fig. 6A), were mutated to Ala in the wild-type α1C and the rEC9 channel, respectively. The mutations increased the IC$_{50}$ values for isradipine 50-fold and 8-fold for the wild-type α1C and the rEC9 channel, respectively (Fig. 7). The fact that this particular Tyr was necessary for the reverse chimera as well as the wild-type α1C channel indicates the essential role of this amino acid residue for the high affinity DHP block.

The present study shows that eight amino acid residues from the α1C subunit, two in IIIS5, three in IIIS6, and three in IVS6, are required to transfer the high affinity DHP sensitivity to the DHP-insensitive α1E Ca^{2+} channel. To our
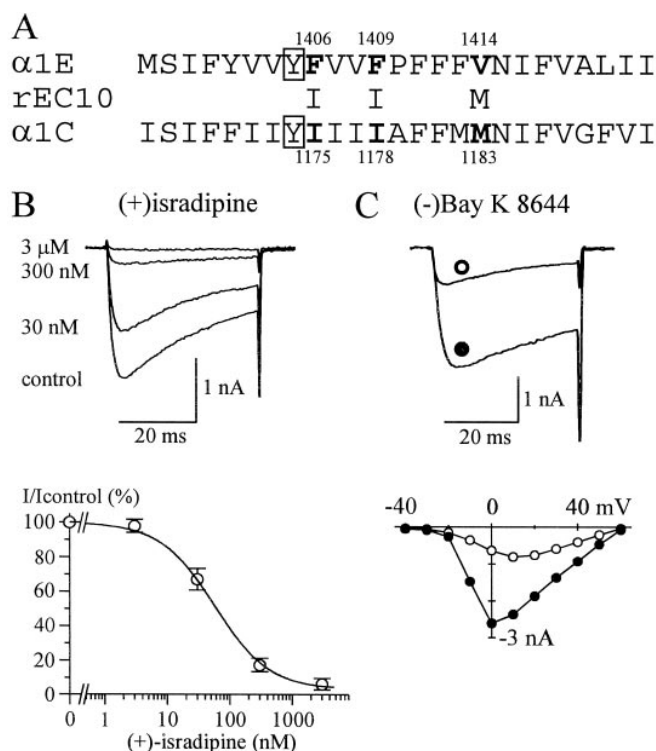
Fig. 6. Contribution of the three amino acid residues in IIIS6 to the DHP sensitivity. A, Amino acid sequence alignment of the IIIS6 for the wild-type and chimeric (rEC10) Ca^{2+} channels. The three amino acid residues indicated were mutated in the reverse chimera rEC9 to construct rEC10. For the contribution of the boxed tyrosines, see Fig. 7. B, Effect of isradipine on the reverse chimera rEC10. The original current traces (top) recorded while superfusing isradipine at the indicated concentrations. The test pulses to +10 mV were applied from the holding potential of −100 mV. The dose-response curve for the drug (bottom) was drawn by the Hill equation with an IC$_{50}$ value of 57.5 ± 2.7 nM (four experiments). C, Effect of Bay K 8644 on rEC10. The current traces (top) and the I-V relations (bottom) obtained before (○) and during (●) the superfusion of 1 μM Bay K 8644. The currents presented (top) were recorded at the test potential of +10 mV (control) and 0 mV (Bay K 8644), which are the potentials showing the peak I-V relation.
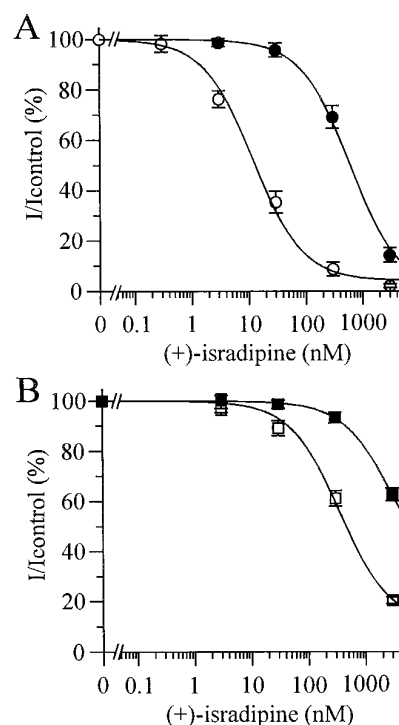

Fig. 7. The DHP blocking effect is modified by the conserved residue Tyr in IIIS6. A, Contribution of Tyr1174 (Fig. 6A) in the wild-type α1C channel to the isradipine sensitivity. The dose-response curves for the wild-type α1C (○) and Y1174A mutant (●) gave the IC$_{50}$ values of 12.2 ± 0.9 nM (11 experiments) and 619 ± 16 nM (six experiments), respectively. B, Contribution of Tyr1405 in the reverse chimera rEC9 to the isradipine sensitivity was studied by the mutation Y1405A. The IC$_{50}$ values for the rEC9 channel (□) and the Y1405A mutant (■) were 0.36 ± 0.03 μM (six experiments) and 3.00 ± 0.29 μM (six experiments), respectively.

knowledge, this is the first report that shows that amino acid residues as few as eight are sufficient for transferring the DHP sensitivity. Among these amino acid residues, Thr1061 in the α1C subunit is essential for the DHP block and stimulation. Its replacement by Tyr completely abolished the DHP sensitivity of both the α1C-containing Ca^{2+} channels and the α1E-containing Ca^{2+} channels. Mutation of Thr1061 in a native DHP-sensitive channel would lead to a loss of the DHP sensitivity, which suggests that care should be taken when Ca^{2+} channels are classified solely by pharmacological criteria. The transfer of the Bay K 8644 stimulation required Gln1065 in addition to Thr1061, strengthening the previous findings (9, 11, 13, 19) that DHP agonists and antagonists require interaction with similar but not identical amino acid residues. How these amino acid residues actually modulate the DHP actions is not clear. Some of the eight amino acid residues responsible for the DHP sensitivity were also shown to be important for the DHP interaction in recent radioligand binding studies (10, 19), which indicates that these residues either are part of the DHP binding site itself or influence the DHP affinity of the binding site. It is also possible that some of these residues are involved in the coupling between the DHP binding and the subsequent conformational change of the Ca^{2+} channel. Replacement of the eight amino acid residues in the α1E subunit (rEC10) dramatically increased its DHP sensitivity, which indicates that a small number of amino acids that are located about halfway between the extracellular and intracellular side of the membrane play a critical role in the DHP actions.

Acknowledgments

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