Structural Determinants of L-type Channel Activation in Segment IIIS6 Revealed by a Retinal Disorder*

Received for publication, June 28, 2005, and in revised form, September 9, 2005 Published, JBC Papers in Press, September 12, 2005, DOI 10.1074/jbc.M507013200

Annette Hohaus1, Stanislav Bey1‡1, Michaela Kudrnan1†, Stanislav Berjukow1, Eugen N. Timin1, Rainer Marksteiner1, Marion A. Maw2¶, and Steffen Hering‡3

From the 1Institute for Pharmacology and Toxicology, University of Vienna, Althanstrasse 14, A-1090 Wien, 2InnoveCell Biotechnology GmbH, Mitterweg 24, A-6020 Innsbruck, and the 3Biochemistry Department, University of Otago, P.O. Box 56, Dunedin 901, Aotearoa, New Zealand

The mechanism of channel opening for voltage-gated calcium channels is poorly understood. The importance of a conserved isoleucine residue in the pore-lining segment IIIS6 has recently been highlighted by functional analyses of a mutation (I745T) in the CaV1.4 channel causing severe visual impairment (Hemarawanu, A., Berjukow, S., Hope, C. I., Dearden, P. K., Wu, S. B., Wilson-Wheeler, J., Sharp, D. M., Lundon-Trewick, P., Clover, G. M., Hoda, J. C., Striessnig, J., Marksteiner, R., Hering, S., and Maw, M. A. (2005) Proc. Natl. Acad. Sci. U. S. A. 102, 7553–7558)). In the present study we analyzed the influence of amino acids in segment IIIS6 on gating of the CaV1.2 channel. Substitution of Ile-781, the CaV1.2 residue corresponding to Ile-745 in CaV1.4, by residues of different hydrophobicity, size and polarity shifted channel activation in the hyperpolarizing direction (I781P > I781T > I781N > I781A > I781L). As I781P caused the most dramatic shift (∼37 mV), substitution with this amino acid was used to probe the role of other residues in IIIS6 in the process of channel activation. Mutations revealed a high correlation between the midpoint voltages of activation and inactivation. A unique kinetic phenotype was observed for residues 779–782 (LA1A) located in the lower third of segment IIIS6; a shift in the voltage dependence of activation was accompanied by a deceleration of activation at hyperpolarized potentials, a deceleration of deactivation at all potentials (I781P and I781T), and decreased inactivation. These findings indicate that Ile-781 substitutions both destabilize the closed conformation and stabilize the open conformation of CaV1.2. Moreover there may be a flexible center of helix bending at positions 779–782 of CaV1.2. These four residues are completely conserved in high voltage-activated calcium channels suggesting that these channels may share a common mechanism of gating.

The entry of Ca2+ through voltage-gated Ca2+ channels has direct effects on muscle contraction, release of hormones and neurotransmitters, hearing, vision, gene expression, and other important physiological functions (2). The pore-forming α-subunits of voltage-gated Ca2+ channels are composed of four homologous domains formed by six transmembrane segments (S1–S6) that are linked together on a single polypeptide (3). A membrane depolarization initiates channel openings (activation) and closures (inactivation). These events can be considered a multistep process consisting of a conformational change in the voltage sensor, a transmission of the signal to the pore region, the opening of the pore, and channel closure due to inactivation. The voltage-sensing machinery is formed by multiple charged amino acids located in segment S4 and adjacent structures of each domain (4). A large number of amino acids involved in Ca2+ channel inactivation have been identified and several molecular mechanisms for this process have been proposed (for reviews see Refs. 5–7).

The molecular mechanism of the voltage-dependent pore opening of Ca2+ channels, however, is less studied and largely unknown. The first attempt to localize the structural elements in Ca2+ channel α-subunits that are involved in channel activation was made by Tanabe et al. (8) who constructed chimeric channels in which sequence stretches of a slow activating (“skeletal muscle-like”) CaV1.1 α-subunit were replaced by sequences from a fast activating (“cardiac-like”) CaV1.2 α1-subunit. The chimeras activated slowly if repeat I of the CaV1.2 α1-subunit was replaced by the CaV1.1 α1-subunit. In a later study, replacement of domains I, II, and III of the low voltage and fast activating CaV3.1 α1-subunit with the corresponding domains of the high voltage-activated CaV1.2 α1-subunit resulted in a high voltage-activated channel (9). An important role of domains I and III but not II and IV on midpoint voltage and time constants of activation was reported by Garcia et al. (10) who mutated the arginines in the S4 segments of all four domains of a chimeric channel to neutral or negative amino acids. The removal of prolines that are conserved in segments IS4 and IIIS4 of voltage-gated Ca2+ channels resulted in shortening of channel open time, whereas introduction of extra prolines to corresponding positions of IIIS4 and IVS4 lengthened the channel open time (11).

Our present study was initiated by the recent finding that a novel retinal disorder is caused by a point mutation (I745T) in segment IIIS6 of the CaV1.4 α1-subunit that shifts the voltage dependence of CaV1.4 channel activation by approximately −30 mV (1, 12). As CaV1.4 channels express only at low density in mammalian cell lines (13) we have decided to study the functional roles of this residue and neighboring residues in segment IIIS6 by introducing and characterizing mutations in the homologous CaV1.2 channel. Our findings demonstrate that residue Ile-781 and three neighboring residues (Leu-779, Ala-780, and Ala-782) play a key role in gating of the CaV1.2 channel.

EXPERIMENTAL PROCEDURES

Mutagenesis—The CaV1.2 α1-subunit coding sequence (GenBankTM X15539) in-frame 3′ to the coding region of a modified green fluorescent protein (GFP)4 was kindly donated by Dr. M. Grabner (14). For

1 Both authors contributed equally to this work.
2 Supported by the Health Research Council of New Zealand.
3 To whom correspondence should be addressed. Tel.: 43-14277-55310; Fax: 43-14277-9553; E-mail: steffen.hering@univie.ac.at.
4 The abbreviations used are: GFP, green fluorescent protein; WT, wild-type; NaChBac, bacterial sodium channel; Bay K8644, 1,4-dihydro-2,6-dimethyl-5-nitro-4-(2-([trifluoromethyl]phenyl)pyridine-3-carboxylic acid methyl ester.

NOVEMBER 18, 2005 • VOLUME 280 • NUMBER 46

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 280, NO. 46, pp. 38471–38477, November 18, 2005 © 2005 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
electrophysiological studies we used the plasmid lacking the GFP tag. Substitutions in segment II6 of the Ca$_{v}$1.2 $\alpha_1$-subunit were introduced by the “gene SOEing” technique (15). In particular, an isoleucine to threonine mutation was introduced in position 781, and further substitutions were made at this position to proline, leucine, alanine, asparagine, glutamine, and arginine. The mutated fragments were cloned into a BamHI-AflII-cassette (nucleotides 1265 and 2689, numbering according to the Ca$_{v}$1.2 $\alpha_1$-subunit coding sequence). This cassette was also used in creation of C769P, G770P, N771P, Y772P, I773P, L774P, L775P, N776P, V777P, F778P, L779P, A780P, and V783P. Mutations that did not lead to functional channels were recloned into the GFP-tagged vector (14) to study membrane targeting. All constructs were checked by restriction site mapping and sequencing.

**Cell Culture and Transient Transfection**—Human embryonic kidney tsA-201 cells were grown at 5% CO$_2$ and 37 °C to 80% confluence in Dulbecco’s modified Eagle’s/F-12 medium supplemented with 10% (v/v) fetal calf serum and 100 units/ml penicillin/streptomycin. Cells were split via trypsin EDTA and plated on 35-mm Petri dishes (Falcon) at 30–50% confluence ~16 h before transfection. Subsequently tsA-201 cells were co-transfected with cDNAs encoding wild-type or mutant Ca$_{v}$1.2 $\alpha_1$-subunits with auxiliary $\beta_2$ ($\alpha_1$) or $\beta_3$ ($\alpha_2$-$\delta$-subunits (18)). The transfection of tsA-201 cells was performed using the FuGENE 6 transfection reagent (Roche Applied Science) following standard protocols.

**Ionic Current Recordings and Data Acquisition**—Barium currents ($I_{\text{Ba}}$) through voltage-gated Ca$^{2+}$ channels were recorded at 22–25 °C using the patch clamp technique (19) by means of an Axopatch 200A patch clamp amplifier (Axon Instruments) 36–48 h after transfection. The extracellular bath solution contained BaCl$_2$ 5 mM, MgCl$_2$ 1 mM, HEPES 10 mM, and choline-Cl 140 mM, titrated to pH 7.4 with methanesulfonic acid. Patch pipettes with resistances of 1–4 megohms were made from borosilicate glass (Clark Electromedical Instruments) and filled with pipette solution containing CsCl 145 mM, MgCl$_2$ 3 mM, HEPES 10 mM, and EGTA 10 mM, titrated to pH 7.25 with CsOH. All data were digitized using a DIGIDATA 1200 interface (Axon Instruments, Inc.) was used for data acquisition and preliminary analysis. The pClamp software package (Version 7.0 Axon Instruments, Inc.) was used for data analysis and curve fitting.

The voltage dependence of activation was determined from current-voltage (I-V) curves that were fitted according to the following modified Boltzmann term: $I = G_{\text{max}} (V - V_{\text{rev}})/(1 + \exp((V_{0.5,\text{act}} - V)/k_{\text{act}}))$ where $V_{\text{rev}}$ is extrapolated reversal potential, $V$ is membrane potential, $I$ is peak current, $G_{\text{max}}$ is maximum membrane conductance, $V_{0.5,\text{act}}$ is voltage for half-maximal activation, and $k_{\text{act}}$ is slope factor. The time course of current activation was fitted to a mono-exponential function: $I(t) = A(\exp(-t/\tau)) + C$ where $I(t)$ is current at time $t$, $A$ is the amplitude coefficient, $\tau$ is a time constant, and $C$ is steady state current. The voltage dependence of $I_{\text{Ba}}$ inactivation (inactivation curve) was measured using a multistep protocol to account for run-down (see Ref. 1). The pulse sequence was applied every 40 s from a holding potential of −100 mV. Inactivation curves were drawn according to a Boltzmann equation: $I_{\text{Ba,inact}} = I_{\text{Ba}} - (I_{\text{Ba}} - I_{\text{Ba,0}})/(1 + \exp((V - V_{0.5,\text{inact}})/k_{\text{inact}}))$ where $V_{0.5,\text{inact}}$ is midpoint voltage, $k_{\text{inact}}$ is the slope factor, and $I_{\text{Ba,0}}$ is the fraction of non-inactivating current. Data are given as mean ± S.E. Statistical significance was assessed with the Student’s unpaired t test.

**Confocal Imaging**—The confocal images were obtained ~30 h after transfection. Data illustrated are representative for 15–20 tsA-201 cells from three independent experiments. Confocal images were acquired with a Zeiss LSM-510 confocal laser scanning microscope, using a 63× (1.4 NA) oil immersion objective. The plasma membrane was stained with 1 µM FM4-64 (amphiphilic styryl dye, Molecular Probes). Images were acquired using an argon laser (excitation, 488 nm; emission BP505–530 nm emission filter) for the GFP-tagged Ca$_{v}$1.2 $\alpha_1$-subunits and a He-Ne laser (excitation, 543 nm; emission filter, LP650 nm) for FM4-64.

**RESULTS**

Mutation I781T Shifts Ca$_{v}$1.2 Channel Activation to More Hyperpolarized Voltages—Residue Ile-745 in segment II6 of the Ca$_{v}$1.4 $\alpha_1$-subunit corresponds to residue Ile-781 in the Ca$_{v}$1.2 $\alpha_1$-subunit (Fig. 1A). The effects of I781T substitution on Ca$_{v}$1.2 channel activation were investigated after expressing wild-type or mutant Ca$_{v}$1.2 $\alpha_1$-subunits together with auxiliary $\beta_2$-$\delta$-subunits in tsA-201 cells. Families of inward Ba$^{2+}$ currents (Fig. 1B) and the corresponding current-voltage (I-V) curves (Fig. 1C) and voltage dependences of channel activation (Fig. 2A) and inactivation (Fig. 2B) are shown. For both wild-type and I781T channels, the current reversed at ~50 mV indicating that the I781T mutation does not affect ion selectivity (Fig. 1C). The midpoint voltage of activation changed from −9.9 ± 1.1 mV in the wild-type channel to −37.7 ± 1.2 mV in the I781T channel (Fig. 2A). I781T also shifted the voltage dependence of inactivation. The midpoint voltage of the inactivation curve changed from −38.7 ± 1.0 mV in wild-type channels to −57.8 ± 0.7 mV in I781T channels (Fig. 2, B and C). Wild-type channels were inactivated by 65 ± 4%, whereas I781T channels were...
Ile-781 Substitutions Showed Slow Activation Gating at Hyperpolarized Voltages and Slow Deactivation—The voltage dependences of the activation time constants for the Ile-781 substitution mutants were also determined (Fig. 3). Between −30 mV and +30 mV, activation of the wild-type and mutant channels occurred with a similar time course (wild-type, $\tau_{\text{act}}$ ranged between 3.2 ms (−30 mV) and 2.5 ms (30 mV); I781P, $\tau_{\text{act}}$ ranged between 3.9 ms (−30 mV) and 2.1 ms (10 mV) (Fig. 3B)). At voltages negative to the threshold potential of wild-type CaV1.2 activation (−40 mV, Fig. 2A), mutant channels displayed decelerated activation kinetics (Fig. 3). At −50 mV, no channel activation was observed in wild-type and I781L (Fig. 3A), whereas the four remaining mutant channels activated at comparatively slow rates (see Fig. 3B for time constants). Moreover, activation of I781P at −60 mV was even slower ($\tau_{\text{act}}$ ~25 ms). In other words, the more the voltage dependence of channel activation was shifted (TABLE ONE), the more slowly the channels activated at hyperpolarized voltages (Fig. 3B).

To gain insight into the stability of the open channel conformation, we also analyzed the voltage dependence of deactivation for I781P and I781T, the mutants that induced the strongest effects on channel activation. The two mutant channels deactivate more slowly than the wild-type channel at all potentials with deactivation of I781P being slower than that of I781T (Fig. 4).

Proline Substitution of Gly-770—A similar kinetic phenotype to that observed for the I781T substitution (slow deactivation, slow activation at hyperpolarized voltages, and decelerated inactivation) was previously observed with other proline substitutions at positions 775, 776, and 779 (Table 1).
reported for a bacterial sodium channel (NaChBac) when a highly conserved glycine (Gly-219) in the central third of S6 was substituted by proline (20). To evaluate an analogous role of Gly-770 in the corresponding position of segment II6 of CaV1.2 (Fig. 1A), we mutated this amino acid to proline. G770P had, however, neither significant effects on the current kinetics (Fig. 5A, second current family from top; Fig. 5B) nor on the voltage dependence of channel activation and inactivation (Fig. 6). N771P adjacent to G770P was found to shift the voltage dependence of channel activation and inactivation (Fig. 6B, TABLE ONE) and to substantially slow the time course of inactivation (Fig. 5A, TABLE ONE). N771P did not, however, show slow channel activation at hyperpolarized voltages ($r_m$ ranged between 1.3 ± 0.2 and 2.9 ± 0.2 ms at all voltages, Fig. 5), and channel deactivation was not decelerated (Fig. 4).

Positional Specificity of I781P—Proline substitution of Ile-781 caused the most dramatic changes in channel activation (Figs. 2–4). To examine the positional specificity of I781P on CaV1.2 channel gating we substituted all amino acids between Cys-769 and Val-783 by prolines (Figs. 5 and 6, TABLE ONE). Substitution of the two alanine residues flanking Ile-781 (A780P and A782P) and of L779P substantially shifted the voltage dependence of activation (Fig. 6, TABLE ONE). A782P and L779P also showed decelerated channel activation at hyperpolarized voltages (Fig. 5B). Additionally channel inactivation was decelerated for A782P and not detectable for A780P and L779P during a 300-ms pulse (Fig. 5A, TABLE ONE). Two peculiarities were observed for A780P. This mutation i) shifted the voltage dependence of channel activation but not inactivation (Fig. 6B), and ii) this mutation slowed the kinetics of channel activation at all voltages (Fig. 5B, inset). Proline substitutions C769P, I773P, and F778P caused smaller or non-significant shifts in the voltage dependence of activation (Fig. 6A, TABLE ONE).

Evidence for Membrane Targeting of Non-functional Mutants—Six constructs (Y772P, L774P, L775P, N776, V777P, and V783P) did not conduct Ba$^{2+}$ currents. To investigate whether the lack of current observed for these mutants could be because of an impairment of plasma membrane targeting, we examined the subcellular distribution of GFP-tagged mutants (14) by confocal microscopy. Wild-type and mutant GFP-tagged CaV1.2 $\alpha_1$-subunits were co-transfected with $\beta_{1a}$ and $\alpha_2$-$\delta_1$-subunits in tsA-201 cells, and the plasma membrane was visualized by staining with FM4-64 (Fig. 7). Consistent with previous reports (21–23), the wild-type CaV1.2 $\alpha_1$-subunit localized predominantly at the plasma membrane with some intracellular labeling also evident. Similarly, images taken from cells expressing the non-functional channels (shown are two representative mutants) demonstrated that all mutant subunits were targeted to the plasma membrane, with some intracellular staining also detected. These findings demonstrate that the lack of current observed for these mutants cannot be attributed to a failure of the mutant GFP-tagged CaV1.2 $\alpha_1$-subunits to reach the plasma membrane.

Bay K8644 and $\beta$-Subunit Modulation of Ile-781 Mutants—A shift of voltage dependence of activation toward more negative voltages is a hallmark of Bay K8644 action. It was therefore interesting to analyze if the shift in activation gating caused by the naturally occurring mutation I781T would affect BAY K8644 action. At potentials corresponding to the maximum of the current voltage relationships (0 mV for WT and −30 mV for I781T, Fig. 1C) 100 nM of BAY K8644 induced similar stimulation (2.3 ± 0.3-fold, $n = 3$ in WT and 2.6 ± 0.3, $n = 3$ in the I781T mutant) (Fig. 8, A and B). The voltage dependencies of channel activation were shifted to comparable extents, −5.9 ± 1.3 mV ($n = 4$) in I781T and −6.7 ± 1.4 mV in WT ($n = 3$) (Fig. 8C).

It is well established that $\beta$-subunits modulate the gating of high voltage-activated Ca$^{2+}$ channels (for reviews see Refs. 24–26). To elucidate whether the observed changes in the voltage dependence of channel activation interfere with $\beta$-subunit modulation, we co-expressed wild-type, I781T, and I781P CaV1.2 $\alpha_1$-subunits with $\beta_{1a}$- or $\beta_{2a}$-subunits as well as the $\alpha_2$-$\delta_1$-subunit and analyzed the resulting activation curves. For each of these channels, co-expression of the $\beta_{2a}$-subunit gave a small hyperpolarizing shift in the voltage dependence of channel activation and slower channel inactivation when compared with co-expression with the $\beta_{1a}$-subunit (Fig. 9, TABLE ONE). These findings...
suggest that these Ile-781 mutants do not substantially affect \( \beta \)-subunit modulation.

**DISCUSSION**

In this study we demonstrate a crucial role of a cluster of amino acids (Leu-779, Ala-780, Ile-781, Ala-782) in the pore-forming segment IIS6 in activation and inactivation of the Ca\(_{\alpha,1.2}\) channel. This finding is related to a recently described channelopathy, where a mutation of the corresponding isoleucine to threonine in Ca\(_{\alpha,1.4}\) (I745T) causes a severe X-linked retinal disorder (12). As previously described for the I745T Ca\(_{\alpha,1.4}\) mutant (1), I781T resulted in an almost identical shift in the voltage dependence of activation (Fig. 1C) and slowed inactivation of Ca\(_{\alpha,1.2}\) (TABLE ONE) suggesting a common mechanism of gating disturbances in both channel types. Additional analyses revealed that the I781T mutation also caused a negative shift in the voltage dependence of inactivation (Fig. 2), slowed activation at hyperpolarized voltages (Fig. 3), and slowed deactivation at all potentials (Fig. 4). These changes in gating of Ca\(_{\alpha,1.2}\) favor transitions to the open state and can be explained by a destabilization of the closed state (i.e. an acceleration of the forward transition to the open state, see 4), a stabilization of the open state (i.e. a deceleration of the backward transition to the closed state, see Ref. 20), or both.

**Ile-781 Substitutions May Destabilize the Closed State**—The five Ile-781 substitutions that produced functional channels caused similar alterations in channel gating (Fig. 2). It seems more feasible to explain...
this finding by a destabilization, rather than by a stabilization, of channel 
conformations. A possible mechanism for destabilization of the closed 
state could be a reduction in hydrophobic interactions in position 781 
with neighboring residues in the S6-bundle crossing region. This expla-
nation is in line with the lower hydrobicity and larger shifts in the 
activation curve of threonine when compared with alanine and leucine 
(Fig. 2A). Thus, hydrophobic interactions of Ile-781 with neighboring 
residues in the bundle crossing region might contribute to stabilization 
of the closed conformation in wild-type channels. Changes in hydro-
phobicity alone can, however, not explain the full picture. Asparagine is 
less hydrophobic than threonine (27), although it causes a similar shift 
(Fig. 2C). As leucine differs from isoleucine by the position of only one 
methyl group and showed the smallest shift, we speculated that Ile-781 
forms part of a tightly fitted region in the closed channel state. Proline 
carried the largest effect on channel activation. Its hydrophobicity is, 
however, closer to the wild-type isoleucine than to threonine and aspar-
agine. Proline might disturb the interaction with neighboring amino 
acids in the closed state by favoring bending of the α-helix (28) and may 
thereby favor an open pore conformation.

Evidence for Stabilization of the Open State—The deceleration of deac-
tivation observed for I781P and I781T (Fig. 4) is a strong indication of a 
stabilization of the open channel conformation. A stabilization of the open 
state is also supported by the reduced rate of channel inactivation that was 
observed for the majority of Ile-781 mutants (TABLE ONE); a prolonged 
sojourn in the open channel state would result in a slower net transition rate 
from the open to the inactivated state. These findings indicate that a point 
mutation in one S6 segment can be sufficient to slow channel closure.

We speculate that closure of voltage-gated Ca^{2+} channels requires 
the return of all four pore-forming elements into the resting state. If one 
element of the gate structure does not precisely fit the resting (closed) 
state, then the conducting pore may remain open for a longer period.

Positional Specificity of Ile-781—To examine the positional specific-
ity of I781P we substituted the residues between Cys-769 and Val-783 by 
prolines (see Fig. 5A). Amino acids causing prominent shifts in the 
voltage dependence of channel activation and inactivation were localized 
in a cluster near the intracellular channel mouth (I781P > L779P, 
A782P, A780P). The more the voltage dependence of channel activation 
was shifted, the slower these channels activated at hyperpolarized 
potentials (Figs. 5 and 6A). A782P showed moderately decreased inac-
tivation, whereas inactivation was severely reduced for L779P and 
A780P suggesting that all three residues contribute to both activation 
and inactivation gating to different extents.

In the upper part of IIS6, N771P caused a shift (−17 mV) of the 
avtivation curve and moderately slowed inactivation, but did not shift 
the inactivation curve markedly and did not affect the rate of channel 
avtivation at hyperpolarized voltages, (Fig. 5B, Fig. 6B, TABLE ONE).
Moreover, unlike I781P and I781T, the voltage dependence of channel 
deactivation was unaltered in this mutant (Fig. 4B). Together these find-
ings suggest that mutation N771P does not stabilize the open state but is 
more likely to destabilize the closed state.

The correlation between shifts in the activation and inactivation 
curves observed for all mutants with the exception of A780P represents 
an interesting finding per se Figs. 2B and 6B. A model describing channel 
avtivation in terms of a voltage-sensing machinery, a discrete voltage-
dependent mechanism of the pore opening and inactivation events 
might reproduce this correlation.

Although correctly targeted to the plasma membrane, some proline 
mutants did not conduct barium currents (Fig. 7). These data suggest that

This content is from the paper "Ca^{2+} Channel Activation Determinants" by W. Rirono, M. V. De Groat, and C. D. Hsueh, published in the Journal of Biological Chemistry, Volume 280, Number 46, November 18, 2005, pages 38471-38476.
proline substitutions in Ca\(^{2+}\) channel S6 segments may disrupt the functionality of the conducting pore. Similar observations were recently made for proline substitutions in the central part of NaChBac segment S6 (29). We cannot exclude, however, that other substitutions in these positions would produce functional Ca\(^{2+}\) channels with altered gating properties.

Bay K8644 and \(\beta\)-Subunit Modulation—Slowing of deactivation and a shift of steady state activation as observed for the naturally occurring mutation I781T and other effective mutations is reminiscent of BAY K8644 action. Interestingly, mutation I781T neither prevented a further shift of channel activation nor affected the stimulation of the current (Fig. 8). These data suggest that Bay K8644 and mutation I781T affect the channel via independent mechanisms. Functional and structural studies suggest that the modulation of channel activation and inactivation by \(\beta\)-subunits occurs by a direct modulation of the movement of the pore-forming segment IS6 (26, 30). The I781P and I781T mutants showed the same shift in the voltage dependence of channel activation and slowed inactivation that occurs when wild-type \(\alpha_1\) subunits are coexpressed with \(\beta_{2a}\) rather than \(\beta_{1a}\) (Fig. 9, TABLE ONE). These findings suggest that \(\beta\)-subunit modulation of segment IS6 and the effects of residue 781 on pore stability are determined by independent mechanisms.

Significance of Ile-781 and Adjacent Residues for Activation Gating of Ca\(^{2+}\) Channels—The crystal structure of the 2-TM helix calcium-gated potassium channel MthK revealed that a conserved glycine at position 83 is responsible for bending the pore-lining M2 helix leading to channel opening (31). Previous studies have shown that the corresponding glycine (Gly-219) in the bacterial voltage-gated sodium channel NaChBac represents a “gating hinge” (20). This conclusion is based on two principle findings, i) G219P shifts the activation curve to hyperpolarized voltages, and ii) this shift is accompanied by a deceleration of deactivation kinetics. Mutating the corresponding Gly-770 in Ca\(_{\text{V1.2}}\) to proline did not cause significant effects on channel gating (Fig. 6, TABLE ONE). Localization of a gating hinge at residue Gly-770 is therefore not likely. The kinetic phenotype of Ile-781 and adjacent residues (Leu-779, Ala-780, and Ala-782) observed in the present study is, however, similar to the one described for NaChBac construct G219P (20). Our data therefore suggest that helix bending is more likely to occur close to the inner channel mouth in Ca\(_{\text{V1.2}}\). Residues 779–782 are conserved in high voltage-gated potassium channel MthK, and it is tempting to speculate that this sequence (LAIA) might participate in a common mechanism of gating. 

Acknowledgments—We thank Dr. Thomas Peterbauer for his support in confocal imaging. The excellent technical assistance of Hannelore Kadlec was greatly appreciated.