The C-terminal Residues in the Alpha-interacting Domain (AID) Helix Anchor CaVβ Subunit Interaction and Modulation of CaV2.3 Channels*

Laurent Berrou§§, Yolaine Dodier§§, Alexandra Raybaud§§, Audrey Tousignant, Omar Dafi§§, Joelle N. Pelletier, and Lucie Parent§§**

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The C-terminal residues in the alpha-interacting domain (AID) helix of high voltage-activated (HVA) CaV2.3 channel α1 subunits binds with high affinity to CaVβ auxiliary subunits. The recently solved crystal structures of the AID-CaVβ complex in CaV1.3/1.2 have revealed that this interaction occurs through a set of six mostly invariant residues Glu/Asp6, Leu7, Gly9, Tyr10, Trp13, and Ile14 (where the superscript refers to the position of the residue starting with the QQ signature doublet) distributed among three α-helical turns in the proximal section of the I-II linker. We show herein that alanine mutations of N-terminal AID residues Glu1, Glu2, Glu4, Glu6, Leu7, and Gly9 in CaV2.3 did not abolish [35S]CaV3-induced modulation but homology was found to be higher within members of the CaV family (Table 1). The AID motif located on the I-II linker of the CaVα1 subunit. The AID motif is absent from LVA T-type (CaV3.1–3.3) VDCC for which CaVβ subunit modulation has never been reported. About half of the residues of the AID helix (Gln1-Gln2-Leu2-Glu4-Tyr10-X11-X12-Trp13-Ile14-X15-X16-X17-Glu18) are strictly conserved (in bold) among the six CaVα1 subunits but homology was found to be higher within members of the CaV-1 and CaV-2 families. Nonetheless, positions 8, 11, and 15 are occupied by residues that could vary even within the same CaV family (Table 1). The AID motif is essential for CaVβ modulation. In vitro binding experiments have revealed the presence of additional interaction sites of lower affinity on the cytoplasmic loops of CaV2.1 (16, 17) and on the C-terminal of CaV2.3 (18) but the AID appears to be the primary high affinity site of interaction (14, 15).

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** To whom correspondence should be addressed. Tel.: 514-343-6673; Fax: 514-343-7146; E-mail: lucie.parent@umontreal.ca.

1 The abbreviations used are: VDCC, voltage-dependent calcium channel; MES, 4-morpholineethanesulfonic acid; r.m.s.d., root mean-squared deviation; GFP, green fluorescent protein; AID, alpha-interacting domain; HVA, high voltage-activated; LVA, low voltage-activated; VDL, voltage-dependent inactivation; SH3, Src homology 3 domain; GK, guanylate kinase.
The recent publication of three crystal structures of the CaV2.3-AID peptide complex provides a unique insight into the intricate set of interactions between the two subunits (19–21). The three-dimensional structures of the conserved core region of CaVα2a and CaVα3, alone and in complex with the AID peptide from CaV1.1 or CaV1.2 channels, show that the CaVα subunit core contains two interacting domains: a Src homology 3 (SH3) domain and a guanylate kinase (GK) domain connected by a HOOK region similar to the one found in PSD95 (19–21). The BID (CaVα interaction domain) is essential both for the structural integrity of the CaVα subunit and for bridging the SH3 domain and GK domains, but does not participate directly in binding the CaVα1 subunit.

The AID peptide from the CaVα1 subunit adopts an α-helical structure upon binding to the CaVα subunit, and the six AID residues from the three main α-helical turns nestle into the hydrophobic groove found in the GK domain opposite to the SH3 domain in the CaVα subunit. The hydrophobic face of the AID helix aptly named the “α-helix-binding pocket” (ABP) contains 7 residues X3, X8, Leu7, Gly9, Tyr10, Trp13, and Ile14 (or Ile387 for the CaVα subunit). The hydrophobic groove is nested into the CaVα subunit at Trp13 (1) and Ile14 play a more critical role than Gly9 and Tyr10. Altogether these results suggest that the C-terminal residues Trp13 (1) and Ile14 play a more critical role than Gly9 and Tyr10 in CaVα subunit functional modulation of HVA Ca2+ channels.

**EXPERIMENTAL PROCEDURES**

**Recombinant DNA Materials—**Standard methods of plasmid DNA preparation were used (29). cDNAs coding for the auxiliary rat CaVβ3 (GenBankTM M88751) and rat brain CaVβ1b (GenBankTM X61394) were kindly donated by Dr. E. Perez-Reyes (30–32). The wild-type human α1E or CaV2.3 (GenBankTM L27745) was a gift from Dr. T. P. Snutch (33). The rat brain CaVα2b subunit was provided by Dr. T. P. Snutch.

**Point Mutations and RNA Transcription—**Point mutations were performed with 40-mer synthetic oligonucleotides using the QuikChangeTM XL-mutagenesis kit (Stratagene, La Jolla, CA). Briefly, mutations were achieved by cassette cloning using the NotI/Xhol CaV2.3 fragment (positions 111 and 1275 nt) subcloned into pBluescript (Stratagene). Constructs were verified by restriction mapping after religation of the mutated fragment into the naturally occurring NotI/Xhol sites of the wild-type CaV2.3. The nucleotide sequence of the mutant channels was confirmed through automated fluorescent DNA sequencing (BioST, La Jolli, Que.). cDNA constructs for wild-type and mutated CaVα1 subunits were linearized at the 3′-end by HindIII digestion whereas the CaVα3 subunit was digested by NotI. Run-off transcriptions were prepared using the methylated cap analog m7Gppp(5′)G and T7 RNA polymerase with the mMessage mMachine® transcription kit (Ambion, Austin, TX). The final cRNA products were suspended in DEPC-treated H2O and stored at −80 °C. The integrity of the final product and the absence of degraded RNA were determined by denaturing agarose gel electrophoresis stained with ethidium bromide.

CaVβ3 and CaVβ1b Overlay Assays onto pGFP-uv Fusion Proteins—A fragment of 105 bp including the whole AID region of CaV2.3 (GenBankTM U62636) (Clontech, BD Biosciences, Mississauga, Ontario) to give a fusion protein with an estimated molecular mass of 32 kDa. The AID-fusion protein was expressed in the chemically competent Escherichia coli strain DH5α. The synthesis of the fusion proteins was induced at 37 °C using 0.5 mM isopropyl-D-thiogalactoside in an overnight liquid culture, and bacteria were collected by centrifugation. As the GFP protein cloned in-frame with the AID3 peptide has been modified to fluoresce under UV light, the fusion protein confers a green color to the DH5α bacterial extracts when monitored under UV light. For overlay assays, crude DH5α bacterial extracts (200 μl) were boiled for 2 min in Laemmli’s loading buffer and separated on a denaturing 10% SDS-polyacrylamide gel. Total proteins were either visualized with Red Protein stain or loaded in duplicate to be visualized by Coomassie staining in addition to the autoradiogram. This half of the gel was transferred onto a polyvinylidene difluoride membrane (Millipore, Fisher, Whitby, Ontario) using Towbin buffer (in mM: 25 Tris, 192 glycine, 20% methanol, and 0.05% SDS). The membrane was blocked with 1% bovine serum albumin in HBS-Tween (in mM: 137 NaCl, 3 KCl, 10 HEPES, and 0.05% Tween-20 pH 7.4), washed once with HBS-Tween and incubated for 1 h at room temperature in 5 ml of HBS-Tween with

The three-dimensional structures vividly account for a wealth of structure-function data gathered over the last decade. Functional studies have long shown the YWI residues to be critical for CaVβ1b and/or CaVβ3 binding in CaV2.1 (24). We have recently shown that point mutations of the conserved Trp13 (Trp386) eliminated the functional modulation of CaV2.3 currents by CaVα3 as well as disrupted the CaVβ3 subunit binding to the AID peptide (1). The stereochemical requirements at this position were strict since substitutions with either hydrophobic (Ala, Gly), hydrophilic (Gln, Arg, Gln), and aromatic (Phe, Tyr) residues led to the same results suggesting that Trp13 (Trp386) played a unique role in CaVα subunit binding and modulation of CaV2.3 channels. Y10F preserved in part CaVβ3 and CaVβ2 binding but preserving the hydroxyl sidechain in Y10S and conserving the aromatic group in the Y10W mutant led nonetheless to a significant decrease in CaVα3, CaVβ3, and CaVβ1b binding to AIDα (CaV2.1) (24, 25). This observation was also confirmed in CaV1.2 and CaV1.1 channels where the Y10S mutant disrupted the plasma membrane localization of the CaVα1 subunit while preserving in part the CaVβ subunit-induced modulation of whole cell currents (26–28). In contrast, point mutations of Glu1, Gly2, Gly4, Leu7, Gly9, and Glu16 failed to abolish the binding of CaVβ3 to the denatured AID peptide from CaV2.1 (13) suggesting that the stereochemical requirements might not be as strict in these positions to achieve a strong interaction with CaVβ subunits. In contrast, there is very little information regarding the role of the N-terminal pair of residues GluLeu in establishing the functional interaction with CaVβ subunits although the crystal structures have demonstrated a strong interaction between the two subunits at this site. It thus remains to be seen whether all of these CaVα-AID-CaVβ interaction sites are required to confer the typical CaVβ subunit-induced functional modulation of CaV1 and CaV2 currents.

Herein we show that the alanine mutation of each of the AID residues Glu1, Glu2, Glu3, Glu4, Leu7, Gly9, and Glu16 did not eliminate [35S]CaVβ1b or [35S]CaVβ3 subunit overlay binding to fusion proteins nor did it prevent the typical modulation of CaV2.3 whole cell currents by CaVβ3. Mutations of Tyr10 with either aromatic (Phe) or positively charged (Arg, Lys) residues yielded whole cell currents that responded to CaVβ3 essentially like the wild-type channel whereas mutations with negatively charged residues (Asp, Gln) disrupted CaVβ3 binding and modulation. The structural requirements were more stringent for Ile14 since the mutation and binding by CaVβ3 were only preserved with the conserved Ileo to Leu mutation. Altogether these results suggest that the C-terminal residues Trp13 (1) and Ile14 play a more critical role than Gly9 and Tyr10 in CaVβ subunit functional modulation of HVA Ca2+ channels.

CaVβ3 and CaVβ1b Overlay Assays onto pGFP-uv Fusion Proteins—A fragment of 105 bp including the whole AID region of CaV2.3 (25-residue AID3 peptide) was generated by polymerase chain reaction and cloned in-frame into the HindIII-KpnI sites of pGFPuv vector (GenBankTM U62636) (Clontech, BD Biosciences, Mississauga, Ontario) to give a fusion protein with an estimated molecular mass of 32 kDa. The AID-fusion protein was expressed in the chemically competent Escherichia coli strain DH5α. The synthesis of the fusion proteins was induced at 37 °C using 0.5 mM isopropyl-β-D-thiogalactoside in an overnight liquid culture, and bacteria were collected by centrifugation. As the GFP protein cloned in-frame with the AID3 peptide has been modified to fluoresce under UV light, the fusion protein confers a green color to the DH5α bacterial extracts when monitored under UV light. For overlay assays, crude DH5α bacterial extracts (200 μl) were boiled for 2 min in Laemmli’s loading buffer and separated on a denaturing 10% SDS-polyacrylamide gel. Total proteins were either visualized with Red Protein stain or loaded in duplicate to be visualized by Coomassie staining in addition to the autoradiogram. This half of the gel was transferred onto a polyvinylidene difluoride membrane (Millipore, Fisher, Whitby, Ontario) using Towbin buffer (in mM: 25 Tris, 192 glycine, 20% methanol, and 0.05% SDS). The membrane was blocked with 1% bovine serum albumin in HBS-Tween (in mM: 137 NaCl, 3 KCl, 10 HEPES, and 0.05% Tween-20 pH 7.4), washed once with HBS-Tween and incubated for 1 h at room temperature in 5 ml of HBS-Tween with
20 μl of [35]S-methionine labeled CaV β3- or CaV β1b subunit prepared as described below. The blots were washed twice for 10 min with 1 mM CaCl2 in HBS-Tween, air-dried, and radioactive signals were detected by autoradiography.

[35]S-Methionine-labeled CaV β3 or CaV β1b in pBluescript (0.5 μg) was synthesized by coupled in vitro transcription and translation (TNT; Promega, Madison, WI) in a 50-μl reaction volume for 1 h, and the reaction mixture was applied without further treatment to the overlay membrane.

Functional Expression of Wild-type and Mutants Channels—Oocytes were obtained from female Xenopus laevis clawed frog (Nasco, Fort Atkinson, WI) as described previously (1, 34, 35). Briefly, stage VI oocytes free of follicular cells were injected with 46 nl of a solution containing 28 ng of cRNA coding for the wild-type or mutated CaV α1 subunit. The CaV α1 subunit was always co-injected with cRNA coding for the rat brain CaV β3 subunit (6) and with or without the rat brain CaV β3 (36) in a 6:2:3 or 6:2:2 weight ratio. The final amount of exogenous CaV β3 injected corresponds to 14 ng, which represents a 23-fold enrichment over the estimated concentration of 0.56 ± 0.02 ng of endogenous CaV β3x2 per oocyte (22). Oocytes were incubated at 19 °C for 1–5 days after injection in Barth’s solution (in mM): 100 NaCl; 2 KCl; 1.8 CaCl2; 1 MgCl2; 10 HEPES; 2.5 pyruvic acid; 100 units/ml of penicillin; 50 μg/ml gentamicin; pH 7.6.

Electrophysiological Recordings in Oocytes—Wild-type and mutant channels were screened at room temperature for macroscopic Ba2+ currents using a two-electrode voltage clamp amplifier (OC-725C, Warner Instruments, Hamden, CT) as described earlier (1, 4, 34). In order to minimize series resistance problems associated with voltage clamping large currents in Xenopus oocytes, experiments were performed on cells with peak currents smaller than ~5 μA in most cases. Hence, the wild-type channel was recorded 3–4 days after cRNA injection using a two-electrode voltage clamp amplifier (OC-725C, Warner Instruments, Hamden, CT) as described earlier (1, 4, 34). Briefly, stage VI oocytes free of follicular cells were injected with 46 nl of a solution containing 28 ng of cRNA coding for the wild-type or mutated CaV α1 subunit. The CaV α1 subunit was always co-injected with cRNA coding for the rat brain CaV β3 subunit (6) and with or without the rat brain CaV β3 (36) in a 6:2:3 or 6:2:2 weight ratio. The final amount of exogenous CaV β3 injected corresponds to 14 ng, which represents a 23-fold enrichment over the estimated concentration of 0.56 ± 0.02 ng of endogenous CaV β3x2 per oocyte (22). Oocytes were incubated at 19 °C for 1–5 days after injection in Barth’s solution (in mM): 100 NaCl; 2 KCl; 1.8 CaCl2; 1 MgCl2; 10 HEPES; 2.5 pyruvic acid; 100 units/ml of penicillin; 50 μg/ml gentamicin; pH 7.6.

Electrophysiological Data Acquisition and Analysis—PCRclamp software, Clampex 6.02 and Clampfit 6.02 (Axon instruments, Foster City, CA) was used for on-line data acquisition and analysis as previously described (1, 34, 35). Unless stated otherwise, data were sampled at 10 kHz and low pass-filtered at 5 kHz using the amplifier built-in filter. For all recordings, a series of voltage pulses were applied from a holding potential of ~80 mV at a frequency of 0.2 Hz from ~−40 to +60 mV. Isochronal inactivation data (h or hinf) were obtained from tail currents generated at the end of a 5-s prepulse (4, 37). Tail current amplitudes were estimated using the function Analyze in Clampfit 6.0 from the peak current arising during the first 10 ms after the capacitive transient (20 data points). Each of these currents was then normalized to the maximum current obtained before the prepulse voltage (I/Imax) and was plotted against the prepulse voltage. For the isochronal inactivation figures,

\[
I/Imax = G_{rel} \times \exp \left( -\frac{zF}{RT} \left( V - E_{0.5,\text{act}} \right) \right)
\]

(Eq. 1)

pooled data points (mean ± S.E.) were fitted to Equation 1 using user-defined functions and the fitting algorithms provided by Origin 6.1 and 7.0 (Microcal Software, Northampton, MA) analysis software. Equation 1 accounts for the fraction of non-inactivating current with \( E_{0.5,\text{act}} \), mid-point potential; \( z \), slope parameter; \( G_{\text{rel}} \), fraction of non-inactivating current; \( V_{m} \), prepulse potential, and \( RT/\phi \) with their usual meanings. The fitting process generated values estimating errors on the given fit values.

Activation potentials were estimated from the normalized I-V curves obtained for each channel combination (22). Although this calculation was not exempt from gating contamination, it provided a qualitative approximation of the CaV β3 modulation on I-V parameters. The I-V relationships were normalized to the maximum amplitude and were fitted to Equation 2, a Boltzmann equation coupled to a linear function.

\[
E_{0.5,\text{act}} \text{ is the potential for 50% activation; } G_{\text{rel}} \text{ is the normalized conductance; } z, \text{ slope parameter; } V_{m}, \text{ the test potential, } V_{\text{trev}}, \text{ the apparent reversal potential and } RT/\phi \text{ with their usual meanings.}
\]

Inactivation kinetics were quantified using R300 values, that is the ratio of the whole cell current remaining at the end of a 300-ms pulse (34, 35, 38). As inactivation kinetics can vary with current density, comparisons between constructs and mutants were generally restricted to whole cell currents lower than ~5 μA as much as possible. Furthermore, this range of current densities made it easier to voltage clamp the oocyte uniformly thus decreasing the possibility of series resistance artifacts contaminating the current kinetics data. Capacitive transients were erased for clarity in the final figures. Statistical analyses and Student’s t test were performed using the fitting routines provided by Origin 6.1 and 7.0 (Microcal Software, Northampton, MA).

Homology Modeling of Wild-type and Mutant CaV2.3—Computations were performed using the InsightII package (version 2000, Accelrys) as described elsewhere (39, 40). Briefly, the BIOPOLYMER and BUILDER modules were used in particular to build or modify molecular structures, and all energy minimizations were performed with the DISCOVER module using the consistent valence force field (CVFF). For all minimizations, one thousand steps of steepest descent minimization were performed, followed by a Conjugate gradient minimization until a convergence of 0.001 kcal/mol/Å was reached.

The starting coordinates were taken from the PDB file 1T0J.pdb (CaV β2a + AID from CaV1.2) (19). Molecular replacement of the following eight residues (L340I, E342R, D343E, K345N, L348R, D349A, T442D, and Q443D) to their reciprocal residues in the human CaV2.3 was undertaken using BIOPOLYMER to obtain the complex CaV β2a + AID wt from CaV2.3. The crystallographic water molecules were not retained. Hydrogen atoms were added using the BIOPOLYMER module at the normal ionization state of the amino acids at pH 7. potentials as well as partial and formal charges were fixed with CVFF. A first series of energy minimizations was then performed with the DISCOVER module using the CVFF with a distance-dependent dielectric constant of 80 (implicit H2O). Minimizations were carried out while keeping the heavy atoms of the protein fixed. The structure obtained was then submitted to a second series of energy minimizations performed as explained above, after removing all constraints on the atoms and with a 20 Å radius sphere of explicit water molecules centered around AID residue Asn92. These two series of minimizations yielded the energy-minimized configuration of CaV β2a + AID wt from CaV2.3. This wild-type configuration was then used to produce six individual mutants: Y437F, Y437R, Y437D, I441L, I441R, and I441D that were also minimized as explained above with a 20 Å radius sphere of explicit water molecules.

RESULTS

A Three-dimensional Model of the AID Peptide from CaV2.3—By homology with the three crystal structures obtained by x-ray diffraction of the AID region in CaV1.1 and CaV1.2 channels, the AID region of the CaV2.3 channel (AID2) should form an α-helix upon binding to CaV β subunits (19–21). The primary sequence of AID2 Gly1-Glu2-Ile3-Glu4-Arg5-Glu6-Leu7-Asn8-Gly9-Tyr10-Ar11-Ala12-Trp13-Ile14-Asp15-Lys16-Ala17-Glu18 conserves 9 out of 18 residues when compared with the AID helix from CaV1.1 and CaV1.2 α1 subunits (Table I). The three-dimensional representations of AID2 obtained by homology modeling with either CaV1.1/CaV β2a (21) (not shown), CaV1.2/CaV β3 (20) (not shown), or CaV1.2/CaV β3a (19) (Fig. 1) all show that AID2 assumes an α-helical structure in the presence of CaV β. The orientation of the residues facing the hydrophilic side of the AID helix (Glu6, Arg4, Arg5, Asn8, Arg11, Ala12, Asp15, and Lys16) was seen to vary slightly but the residues mostly located on the hydrophobic face of the AID2 helix that includes the invariant LGWYI residues (Glu6, Leu7, Gly3, Tyr10, Trp13, and Ile14) are well buried in the CaV β subunit fold. As seen in Fig. 1, the front (A), the back (B), and the bottom (C) perspectives of the AID2 model obtained using the crystal coordinates of CaV1.2/CaV β2a (19) clearly show that the side-chains of Tyr10, Trp13, and Ile14 are entrapped within...
the Caβ subunit. Hence, the C-terminal residues appear to interact extensively with the Caβ subunit. The Gly9 residue is significantly enclosed by the Caβ subunit and could be needed to provide some degree of flexibility to the AID helix. The crystal structures further show that the aromatic side-chain of Tyr10 is significantly surrounded by residues of the Caβ subunit. The Gly9 residue is color-coded: yellow, L380 (side-chains on the hydrophobic face of the helix are color-coded: yellow, red, green, blue, magenta, light blue, pink). The N-terminal alanine mutants displayed the additional N-terminal residues Glu6 and Leu7 appear to form fewer interactions with Caβ suggesting these three positions might be significantly larger at 0.05. By comparison, the R300 values obtained for W386A (WA) mutant failed to interact with either [35S]Caβ1β or [35S]Caβ3. W386A mutant was not either modulated by Caβ1β (1) or Caβ1β (not shown).

As seen in Fig. 2, D–G, the binding data correlated well with the functional data. The six alanine mutants were expressed in Xenopus oocytes in the presence and in the absence of Caβ3. Under both conditions, the N-terminal mutants yielded robust inward Ba2+ currents with current-voltage relationships typical of voltage-gated Ca2+ channels. Typical current traces are shown for E6A (E379A) and L7A (L380A) in the absence (left panels) and in the presence (right panels) of Caβ3. To ensure that the current density was not unduly influencing the VDI (voltage-dependent inactivation) gating, the RNA concentrations were adjusted to yield peak currents in the -3 to -5 μA range when possible. Co-expression with Caβ3 slightly hyperpolarized the activation potential for I377A, E379A, and L380A at p < 0.01 and had little or insignificant effect on Q374A and G376A (see Table II for detailed values). The VDI gating was then estimated from the whole cell currents remaining after 100 ms (R300). In the absence of Caβ3, the R300 values were similar for most channels (wild-type and mutants) although they were significantly larger at p < 0.001 for the N-terminal mutants Gln1, Gln2, Ile3, and Glu4 (Fig. 2F) suggesting that the N-terminal end of the AID helix could be an intrinsic determinant of VDI gating in this channel (44). Co-injection with Caβ3 significantly decreased the R300 values for the wild-type channel, Q374A, Q375A, I376A, E377A, and E379A with less than 10% of the whole cell currents remaining at 300 ms (Fig. 2G). Hence, the VDI gating of N-terminal mutants was not significantly different from the wild-type channel (p > 0.05). L380A turned out to be the only mutant with kinetics slightly significantly faster than the wild-type channel at p < 0.05. By comparison, the R300 values obtained for W386A under the same conditions were 3-times larger, with roughly 30% of the whole cell currents remaining at the end of a 300-ms pulse and significantly different from the wild-type channel at p < 10-10. Altogether, these data indicate that the VDI gating of the alanine mutants was intrinsically similar to the wild-type channel in the absence of Caβ3 and further show that they were modulated by Caβ3 in a wild-type fashion.

The N-terminal alanine mutants displayed the additional overlay binding assays for point mutations Q1A, Q2A, 13A, E4A, E6A, L7A performed in the 18-residue AID peptide from Caβ2.3 (AIDβ) inserted in the pGFPuv vector that corresponds respectively to mutants Q374A, Q375A, I376A, E377A, E379A, and L380A in Caβ3. Caββ subunit binding was evaluated with both [35S]methionine-labeled Caβ3 (Fig. 2B) and Caβ1β (Fig. 2C) subunits. As seen, strong signals were obtained in both cases at the expected molecular mass of 32 kDa for the wild-type AID peptide ~ Q374A ~ Q375A ~ I376A ~ E377A ~ E379A > L380A mutants indicating that both Caβ3 and Caβ1β interacted strongly with the alanine mutants under denaturing conditions. In contrast, the empty pGFPuv vector (29 kDa) and the well documented W386A (WA) mutant failed to interact with either [35S]Caβ1β or [35S]Caβ3. W386A mutant was not either modulated by Caβ1β (1) or Caβ1β (not shown).

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The N-terminal alanine mutants displayed the additional
Role of C-terminal Residues in the AID Helix

Fig. 2. Alanine mutations in the N-terminal end of the AID helix do not impede CaV-β binding and modulation. A, primary sequence of the AID region (Q1 Q2 I3 E4 E5 E6 L7 N8 G9 Y10 R11 A12 W13 I14 D15 K16 A17 E18) is shown for CaV2.3 (AID). The N-terminal residues mutated in this figure are underlined. B, autoradiogram of in vitro translated [35S]CaV-β3 overlays on GFPuv-AIDE mutants immobilized on nitrocellulose. Strong signals were detected (from left to right) for the wild-type AID peptide, Q374A (Q1A), Q375A (Q2A), I376A (I3A), E377A (E4A), E379A (E6A), and L380A (L7A). [35S]CaV-β3 binding could not be detected for the empty vector pGFPuv and the AID mutant W386A (WA). C, autoradiogram of in vitro translated [35S]CaV-β1b overlays on GFPuv-AIE mutants immobilized on nitrocellulose. Strong signals were detected (from left to right) for the wild-type AIE peptide, Q374A (Q1A), Q375A (Q2A), I376A (I3A), E377A (E4A), E379A (E6A), and L380A (L7A). [35S]CaV-β3 binding could not be detected for the empty vector pGFPuv and the AID mutant W386A (WA). D, G, typical whole cell current traces obtained after the expression of the E379A (E6A) mutant in Xenopus oocytes with CaVα2b without exogenous CaV-β3 (+β3) and after co-injection with CaV-β3 (+β3). Time and current scales are identical for D and E, E, typical whole cell current traces obtained after the expression of the L380A (L7A) mutant in Xenopus oocytes with CaVα2b without exogenous CaV-β3 (+β3) and after co-injection with CaV-β3 (+β3). F, mean R300 ratios (the fraction of the whole cell current remaining at the end of a 300-ms pulse) are shown ± S.E. from 0 to +20 mV for the wild-type and the mutant CaV2.3 channels (Q374A, Q375A, I376A, E377A, E379A, L380A, and W386A) in the absence of CaV-β3. G, R300 graph for the wild-type and the mutant CaV2.3 channels (Q374A, Q375A, I376A, E377A, E379A, L380A, and W386A) after co-injection with CaV-β3. As seen, the VDI kinetics of the N-terminal mutants are significantly accelerated in a manner comparable to the wild-type channel. In contrast, the kinetics of W386A were not increased by CaV-β3 suggesting that the first turn of the AID helix is more tolerant to alanine mutations. Whole cell currents were recorded using the two-electrode voltage clamp technique in the presence of 10 mM Ba2+ after injection of EGTA. Holding potential was −80 mV. Xenopus oocytes were pulsed from −40 mV to +60 mV using 10 mV steps for 450 ms. All mutants expressed significant inward currents with typical current-voltage properties. Capacitive transients were erased for the first ms after the voltage step. The mutant properties (mid-potentials of activation and inactivation as well as peak currents) are provided in Table II.

hallmarks of CaV-β-modulation. Co-expression with CaV-β3 significantly hyperpolarized by −30 mV the mid-potential of inactivation of the N-terminal mutants replicating the behavior of the wild-type channel from a E0.5,inact = −36 ± 3 mV (10) in the absence of CaV-β to E0.5,inact = −64 ± 1 mV (10) with CaV-β3 as previously published (1, 4, 34) (Table II). Considering that the fifth position (Arg278) was previously studied and reported (1, 34), these data indicate that alanine substitutions at any of the first seven positions of the AID6 helix failed to alter significantly CaV-β subunit binding and modulation of CaV2.3 channels. Hence, the N-terminal Glu6 and Leu7 residues located in the first α-helical turn of the AID6 helix are tolerant to alanine substitutions.

Ala nine Scan of the GY1 Residues in the o-Helix C-terminal Turns in CaV2.3—The role of the two remaining pairs of critical residues Gly9/Tyr10 and Trp13/Ile14 located on the C-terminal α-helical turns was also investigated after mutation with alanine. As explained earlier, CaV-β subunit binding was investigated in overlay assays whereas modulation by CaV-β subunits was studied after functional expression in Xenopus oocytes. Fig. 3A shows the overlay binding assays for Y383A, G382A, and I387A performed in the 25-residue AIDE peptide inserted in the pGFPuv vector. CaV-β3 subunit binding is shown for [35S]methionine-labeled CaV-β1b (Fig. 3A). The empty pGFPuv vector and wild-type AID6 peptide were tested as additional controls. Strong bands were obtained at the expected...
Biophysical properties of CaV2.3 mutants

Biophysical parameters of CaV2.3 wild-type and mutant channels expressed in Xenopus oocytes in the presence of CaVα2βδ and CaVβ3 subunits. Whole cell currents were measured in 10 mm Ba⁺⁺ throughout. The voltage dependence of inactivation was determined from the peak currents measured at 0 mV after 5-s pulses from +100 to +50 mV. Relative currents were fitted to Boltzmann equation 1. The fractional currents represent the fraction of whole cell currents remaining at the end of a 5-s conditioning pulse to +10 mV. Activation data were estimated from the mean I-V relationships and fitted to Boltzmann equation 2. Peak I_{th} was determined from I-V relationships for the corresponding experiments. The data are shown with the mean ± S.E.

| αβδ in Ba | Inactivation (mV) E_{inact} | Activation E_{act} | Peak I_{th} | pGFPuv binding |
|-----------|---------------------------|-------------------|-------------|---------------|
|           | −β            | +β                 | −β          | +β            | −β          | +β          |
| Wild type | −36 ± 3 (10)   | −64 ± 1 (10)       | −10 ± 1 (10) | −1.5 ± 0.3 (10) | +3 ± 4 (10) | ++          |
| Q374A     | −37 ± 1 (5)    | −61 ± 1 (9)        | −13 ± 1 (5)  | −4.9 ± 0.7 (5)  | −6 ± 1 (5)  | ++          |
| Q375A     | −37 ± 1 (5)    | −63 ± 1 (12)       | −10 ± 2 (4)  | −7 ± 2 (6)      | −4 ± 2 (4)  | ++          |
| I376A     | −34 ± 1 (9)    | −61 ± 1 (10)       | −8 ± 2 (14)  | −1.1 ± 0.1 (3)  | −3.7 ± 0.8 (14) | ++      |
| E377A     | −35 ± 1 (9)    | −62 ± 1 (13)       | −11 ± 2 (11) | −3.0 ± 0.4 (11) | −2.5 ± 0.1 (3) | ++      |
| G382A     | −37 ± 1 (8)    | −67 ± 1 (3)        | −9 ± 1 (4)   | −4.9 ± 0.5 (9)  | −2.2 ± 0.1 (4) | ++      |
| I387A     | −34 ± 1 (12)   | −61 ± 1 (11)       | −12 ± 1 (11) | −3.6 ± 0.3 (12) | −5.0 ± 0.7 (11) | +        |
| G382A     | −43 ± 1 (3)    | −66 ± 1 (4)        | −10 ± 2 (12) | −1.7 ± 0.2 (3)  | −3.0 ± 0.5 (12) | ++      |
| Y383A     | −50 ± 1 (8)    | −54 ± 1 (10)       | −10 ± 1 (7)  | −3 ± 1 (8)      | −2.9 ± 0.4 (7) | −        |
| Y383S     | −26 ± 1 (7)    | −46 ± 1 (13)       | −6 ± 2 (5)   | −2 ± 1 (4)      | −1.4 ± 0.4 (11) | −        |
| Y383R     | −35 ± 1 (6)    | −65 ± 1 (10)       | −6 ± 1 (10)  | −5 ± 1 (8)      | −1.8 ± 0.4 (10) | +        |
| Y383K     | −33 ± 1 (16)   | −61 ± 1 (15)       | −12 ± 1 (14) | −4 ± 1 (16)    | −4 ± 1 (14) | +        |
| Y383E     | −33 ± 1 (9)    | −36 ± 1 (19)       | 0.3 ± 0 (3)  | 0.2 ± 0.1 (3)   | −3.7 ± 0.7 (9) | −        |
| Y383D     | −30 ± 1 (8)    | −40 ± 1 (8)        | −9.5 ± 0.4 (8) | −4.0 ± 0.2 (11) | −5.6 ± 0.6 (8) | −1.8 ± 0.2 (11) | +        |
| Y383F     | −33 ± 1 (5)    | −64 ± 2 (6)        | −7 ± 2 (5)   | −3.9 ± 0.9 (5)  | −2.6 ± 0.4 (5) | +        |
| Y383W     | N/E           | N/E                | N/E         | N/E          | N/E        | −        |
| W386A     | −39 ± 2 (7)    | −35 ± 1 (6)        | −7 ± 1 (7)   | −7 ± 1 (7)     | −1.6 ± 0.9 (7) | −2.1 ± 0.8 (6) | −        |
| I387A     | −33 ± 1 (17)   | −46 ± 1 (13)       | −9 ± 1 (12)  | −2.2 ± 0.5 (19) | −2 ± 2 (12) | −        |
| I387L     | −40 ± 1 (10)   | −63 ± 1 (6)        | −8 ± 1 (20)  | −3.5 ± 0.2 (20) | −2.7 ± 0.4 (22) | n.d.  |
| I387S     | −35 ± 2 (23)   | −54 ± 1 (19)       | −9 ± 1 (17)  | −2.8 ± 0.7 (17) | −1.3 ± 0.2 (10) | −        |
| I387E     | −32 ± 1 (4)    | −42 ± 1 (11)       | −8 ± 1 (7)   | −1.0 ± 0.1 (11) | −3.0 ± 0.8 (7) | −        |
| I387D     | −32 ± 1 (10)   | −31.8 ± 0.7 (6)    | −7 ± 1 (24)  | −4 ± 1 (9)     | −9 ± 1 (5) | −        |
| I387K     | −31.7 ± 0.6 (5)| −37 ± 0.4 (10)     | −6 ± 1 (11)  | −2.4 ± 0.3 (8)  | −3.4 ± 0.6 (11) | −        |
| I387R     | −36 ± 1 (15)   | −35 ± 2 (3)        | −9.2 ± 0.4 (16) | −2.7 ± 0.4 (16) | −2.6 ± 0.5 (9) | −        |
| E391A     | −35 ± 1 (6)    | −62 ± 1 (3)        | −12 ± 0.3 (14) | −8 ± 2 (9)     | −2.0 ± 0.4 (14) | ++      |

* The number of samples appears in parentheses.

** N/E stands for non-expressor.

† Not determined.

molecular weight of 32 kDa for the wild-type AID_{β} peptide as well as for G382A indicating that the alanine mutation at position Gly^{a} did not impede the interaction with [^{35}S]CaV_{β3}b. Similar results were obtained with [^{35}S]CaV_{β3}b (not shown). The binding data were compared with the electrophysiological data obtained for the same mutants in the presence and in the absence of CaV_{β3}. As expected from the binding studies, CaV_{β3} modulated the whole cell currents of G382A in a fashion reminiscent of the wild-type channel (Fig. 3B) with a ~5-fold decrease in the R300 values in the presence of CaV_{α2β3} (p < 10^{-5}) (Fig. 3D). Table II reports the estimated mid-potentials of activation and inactivation under the same conditions. Although alanine mutations to Tyr^{10} or Ile^{14} positions were sufficient to upset the binding of [^{35}S]CaV_{β3}b to the AID_{β} peptide, CaV_{β3}-induced modulation of the Y383A and I387A channels was mostly preserved in Y383A and partially preserved with I387A (Fig. 3C). Co-expression with CaV_{β3} significantly decreased the R300 values of Y383A at p < 10^{-4} at all membrane potentials in a wild-type fashion (Fig. 3, E and F). The functional modulation of CaV_{2.3} Y383A by CaV_{β3} has already been reported before by us (1). The mid-potential of
inactivation was also hyperpolarized by CaVβ3 although the
−20 mV shift was significantly smaller at p < 10−4 than the
−30 mV routinely observed for the wild-type channel (Table II).
The data for I387A appeared to be more easily reconciled with
the binding studies. The modulation of VDI gating kinetics as
measured at 300 ms was milder for I387A although the R300
values measured with CaVβ3 remained significantly smaller
(p < 0.05 at 0 mV) (Fig. 3F). The milder modulation of the
I387A mutant by CaVβ3 was also apparent in the smaller shift
in the voltage dependence of inactivation with E_{0.5,inact} experi-
encing a −13 mV shift in average as compared with the −30
mV displacement reported for the wild-type channel (Table II).
Hence, measuring CaVβ binding to the AID peptide under
denaturing conditions does not appear to entirely account for
the interaction between the I-II linker and CaVβ subunits.

Aromatic and Positively Charged Residues at Tyr^{10} Preserve
CaVβ Binding and Modulation—Because the substitution of the
aromatic tyrosine residue Tyr^{10} at position 383 by the small
and hydrophobic alanine (Ala) residue was shown to disrupt
CaVβ3 subunit binding as well as decreasing the CaVβ-induced
modulation of CaV2.3, the structural requirements for CaVβ
subunit binding and modulation were further investigated af-
ter substitutions with aromatic (Phe, Trp) as well as positive
(Arg, Lys) and negative (Asp, Glu) residues. CaVβ subunit-
induced modulation of inactivation was also preserved with the
Y10S mutant in L-type CaV1.1 and CaV1.2 channels (26)
whereas in vitro binding of CaVβ4 to AID_{x} was demonstrated
in the presence of the Y10S and Y10F mutants (25). We had
previously reported that the substitution with the neutral but
lighter serine (Ser) residue at the same position yielded a chan-
nel with VDI gating slower than the wild-type channel in the
presence of CaVβ3 (1). Nonetheless, the voltage dependence of
inactivation of Y383S was clearly shifted in the hyperpolarized
direction by −20 mV in the presence of CaVβ3 (1). These data
were replicated over a 2-year period, and Table II provides the
complete set of numerical values for this and the other
Tyr^{10} mutants.

[^{35}S]CaVβ1b binding to pGFPuv-AID_{x} fusion proteins mu-

![FIG. 3. CaVβ subunit binding and modulation of G382A, Y383A, and I387A channels.](image)
tated to Y383F, Y383E, Y383K, and Y383R is shown in Fig. 4A whereas [35S]CaVβ3 binding to pGFPuv-AID2 mutants Y383F, Y383S, Y383E, Y383K, and Y383W in Fig. 4B. For the CaVβ1b data, the wild-type AID2 fusion protein was used as a positive control whereas W386A was loaded to assess the background signal. E391A (E18A) was used as a positive control in the presence of CaVβ3 since it remained functionally modulated by CaVβ3 in a wild-type fashion (Table II). Altogether, the signal intensity for the AID2 fusion peptides ranked from AID2 wt > E391A, > Y383F > Y383W > Y383K > Y383R > Y383S > W386A. The binding signal for Y383E was somewhat stronger in the presence of CaVβ3. As seen, the signal was similarly for the two aromatic mutations Y383F and Y383W confirming that the aromatic side-chain is a key structural requirement. Furthermore, positively charged residues were more tolerated than the negatively charged ones suggesting that electrostatic-based interactions are important for [35S]CaVβ1b and [35S]CaVβ3 binding at least under our experimental conditions where the AID helix has been denatured.

To establish a functional correlation with the binding studies, Tyr383 mutants (Phe, Trp, Ala, Lys, Arg, Ser, Asp, Glu) were expressed ± CaVβ3 and characterized by the double electrode voltage clamp approach in Xenopus oocytes. All Tyr383 mutants, with the exception of Y383W, expressed robust inward currents in the presence of 10 mM Ba2+ (Fig. 4, C and D). The activation potentials of the mutant channels were compa-
rable to the wild-type channel although their mean current-voltage relationships were not significantly shifted by CaVβ3 in the hyperpolarized direction (Table II). The VDI kinetics (Fig. 4, E and F) and voltage dependence of inactivation (Table II) of Tyr383S mutants were modulated by CaVβ3 in a manner that mimics the binding data. The R300 values plotted as a function of voltage indeed revealed that substitutions with aromatic (Phe), neutral (Ala), and positively charged (Arg, Lys) residues reproduced the phenotype of the wild-type channel shown as a control (Fig. 4E). CaVβ3 compellingly increased the VDI gating kinetics of these mutants with a 4-fold acceleration that is similar to the wild-type channel (p < 10−5). Furthermore, the voltage dependence of inactivation of these mutants was indistinguishable from the wild-type channel whether measured in the presence or in the absence of CaVβ3 with the exception of Y383A that inactivated at E0.5 inact = −54 ± 1 mV (10) in the presence of CaVβ3 (Table II). In contrast, mutations with the negatively charged aspartate and glutamate residues eliminated the functional modulation of VDI gating (Fig. 4F) and blunted the CaVβ3-induced hyperpolarizing shift in the voltage dependence of inactivation (Table II). Mutation with the neutral but polar serine residue significantly restrained the extent of CaVβ3-induced modulation. Altogether, it can be concluded that the Tyr10 position in the second turn of the AID helix is relatively tolerant to substitutions with aromatic, small hydrophobic, or positively charged residues.

The Iso to Leu Substitution Is Tolerated at Position Ile14—The stereochemical requirements for CaVβ binding and modulation at position Ile14 were investigated after substitutions with the conserved leucine residue (Leu), the neutral but polar serine (Ser) as well as positive (Lys, Arg) and negative (Asp, Glu) residues. As seen earlier, the substitution of the hydrophobic isoleucine residue at this position by the likewise hydrophobic but smaller alanine residue was shown to disrupt CaVβ3 subunit binding as well as decreasing the CaVβ3-induced modulation of CaV2.3 suggesting that the volume of the residue plays a critical role in establishing essential interactions with CaVβ subunits.

[35S]CaVβ1b binding to pGFPuv-AIDk fusion proteins mutated to I387D, I387R, and I387K is shown in Fig. 5A. As shown before, the wild-type AIDk fusion protein and W386A were used as positive and negative controls, respectively. The signal was unambiguous for AIDk wt but was indistinguishable from the background noise for I387D ~ I387R ~ I387K ~ W386A. Similar data were obtained with [35S]CaVβ3 with I387S ~ I387D ~ I387E ~ I387R ~ I387K (not shown). As seen, none of the polar mutations at this position, including the mutation by the neutral serine, preserved binding to [35S]CaVβ under denaturing conditions.

The functional characterization was carried out as discussed before in the presence of CaVβ3. All the Ile14 mutants activated within the same voltage range as the wild-type channel (Table II). In agreement with the binding data, the CaVβ3-induced modulation of VDI kinetics (Fig. 5, B–E) and voltage dependence of inactivation (Table II) was preserved to a certain extent after substitution with neutral residues but was lost with the charged substitutions. Typical current traces are shown for I387L ± CaVβ3 (Fig. 5F) and I387K ± CaVβ3 (Fig. 5C). As shown in the R300 graph, the inactivation kinetics of I387L, I387A, I387S, and the wild-type channel were not significantly different in the absence of CaVβ3 (p > 0.05) (Fig. 5D). Although co-injection with CaVβ3 sped up to a certain extent the inactivation kinetics of the three neutral mutants (Leu, Ser, Ala), I387A + CaVβ3 and I387S + CaVβ3 remained significantly slower at p < 10−5 than the wild-type and the I387L channel measured under the same conditions (p < 10−5) suggesting that the functional interaction with CaVβ3 was fully preserved with the conserved I387L mutation but not optimal with the I387A and the I387S mutants.

Substitutions with positively charged (Arg, Lys) or negatively charged (Asp, Glu) residues yielded functional channels with typical I-V properties that were ineffectually modulated by CaVβ3 (Fig. 5E). Although CaVβ3 appeared to significantly speed up the VDI kinetics of I387E at 0 mV (p < 10−5), this effect vanished at +20 mV. Furthermore the R300 values for I387E + CaVβ3 were systematically larger than the wild-type channel under the same conditions. Finally, the voltage dependence of inactivation of the charged mutants was not significantly hyperpolarized by the co-injection with CaVβ3 (Table II). Altogether these data suggest that the position Ile14 specifically requires a bulky, neutral, and hydrophobic residue to achieve CaVβ3-induced modulation of CaV channels.

DISCUSSION

The C-terminal Turn of the AID Helix Anchors the Interaction with CaVβ—The four known auxiliary CaVβ (CaVβ1–4) subunits can potentially associate with any of the six CaVα1 pore-forming subunits of HVA VDCC (CaV1.1–1.3; CaV2.1–2.3) via AID located on the II-1 linker of the CaVα1 subunit. About half of the residues of the AID helix Glu1, Glu2, Glu6, X–X–X–X–Leu–X–X–Gly3–Tyr6–X11–X–X–X–Trp13–Ile14–X5–X–X–X–Glu18 (19) are strictly conserved among the six CaVα1 subunits but homology was found to be higher within members of the CaV1 and CaV2 families (Table I). The crystal structures of the CaVβ/AID peptide complex from L-type CaVα1.1 and CaVα1.2 channels have recently revealed that the AID region forms an α-helix upon binding CaVβ subunits (19–21). The interaction between the AID helix and the CaVβ protein appears to be primarily secured by the projection of three pairs of hydrophobic residues onto the CaVβ fold with Tyrr15 and Trp15 being almost completely buried within the CaVβ protein (19–21). The three-dimensional model of AIDk obtained by homology modeling with CaV1.2/CaVβ2a (19) suggests similarly that the α-helical structure of the AID peptide is preserved in CaV2.3 with the three pairs of residues Glu6/Leu7, Gly7/Tyr10, and Tyr13/Ile14 projecting into CaVβ residues with Leu7, Gly7, Tyr10, and Ile14 being invariant in all HVA CaVα1 channels. The sixth position is always occupied by a negatively charged residue with the aspartate residue in CaV1.1 being replaced by a glutamate residue in CaV2.2 channels. The three-dimensional model of AIDk shows that Tyr10 and Trp13 are deeply enmeshed within residues of the CaVβ subunit suggesting that these residues could play a more critical role in CaVβ modulation of CaV2.3 currents than Glu1, Gly2, Ile3, Glu4, Glu6, Leu7, Gly9, and Ile14.

Our mutational analysis herein demonstrated that alanine mutants of Glu1, Glu2, Ile3, Glu4, and Glu18 did not abolish [35S]CaVβ1b or [35S] CaVβ3 subunit overlay binding to fusion proteins nor did they prevent the typical modulation of CaV2.3 whole cell currents by CaVβ3. These results suggest that the N-terminal residues of the AID helix located on the hydrophilic face of the AID helix do not play a determinant role in the interaction between CaVα1 and CaVβ subunits in agreement with the predictions of the crystal structure. In general, the signal intensity to the AIDk peptide was found to be more robust for [35S]CaVβ1b than for [35S]CaVβ3 binding in agreement with previous reports showing that CaVβ1b displayed a significantly higher affinity for the AIDk peptide than CaVβ3 in CaV2.1 channels (17, 41).

Furthermore, alanine mutations of Glu6 and Leu7 located in the first hydrophilic α-helical turn of the AIDk helix did not affect significantly CaVβ binding and modulation of CaV2.3 currents. These results suggest that the binding pocket at
these positions can accommodate alanine residues. The central α-helical turn of the AID helix was slightly less tolerant to mutations than the N-terminal residues. In fact, mutation of Gly by alanine enhanced CaVβ-induced modulation indicating that the helical propensity conferred by the alanine residue fastened the interaction with CaVβ whereas increased flexibility at this position could loosen the interaction. Mutations of Tyr with either aromatic (Phe) or positively charged (Arg, Lys) residues yielded whole cell currents that responded to CaVβ essentially like the wild-type channel whereas mutations with negatively charged residues (Asp, Glu) disrupted CaVβ binding and modulation.

In contrast, mutations of the C-terminal residues Trp13 and Ile14 disrupted significantly CaVβ binding and modulation. We had previously provided evidence that position Trp13 could not be successfully substituted with hydrophobic (Ala, Gly), hydrophilic (Gln, Arg, Glu), or aromatic (Phe, Tyr) residues (1). Herein, we further demonstrate that modulation and binding by CaVβ was significantly weakened in I14A (neutral and hydrophobic) and I14S (neutral and polar) mutants and completely eradicated in negatively charged I14D and I14E or positively charged I14R and I14K mutants. The conservative mutation I14L was found to be the only substitution that preserved the CaVβ-induced modulation of CaV2.3 currents. Hence, in contrast to the N-terminal residues, we have compellingly established that CaVβ binding as well as CaVβ-induced modulation of HVA CaV currents are determined by the C-terminal pair of residues of the AID helix.

**Computer Modeling and Molecular Replacements of Tyr**

**Tyr**

The analysis of the accessible surface area in the CaV1.2 AID-CaVα complex showed that the binding pocket formed at the Tyr position could impose harsher structural requirements than the binding pocket at Ile14 (19). On the contrary, our data revealed that mutations at position Ile14 impacted more significantly on the environment caused by the AIDE mutations, molecular replacements were produced in the three-dimensional model of
the wild-type AID$_h$ helix obtained for CaV$_2.3$ (see "Experimental Procedures"). The resulting energy-minimized configuration was then compared with the three-dimensional model for the wild-type AID$_h$ helix.

The three-dimensional model of the AID$_h$ helix implies that Ile$_{14}$ could make van der Waals interactions with CaV$_{\beta 2a}$ hydrophobic residues away from the AID$_h$ helix and results in a significantly increased r.m.s.d. value (1.53 Å as compared with 0.55 Å for I14L) for the four hydrophobic CaV$_{\beta 2a}$ residues. The BUILDER module was used to construct the molecular replacements onto the CaV$_2.3$ model and all energy minimizations were carried out with the DISCOVER module as described under "Experimental Procedures."

In contrast with the I14R mutation, our molecular replacement experiments suggest that the introduction of an aspartate residue is not likely to impact significantly on the position of the CaV$_{\beta 2a}$ residues as a result of steric constraints. Indeed, the r.m.s.d. values for the AID$_h$ helix (0.12 Å) as well as for the CaV$_{\beta 2a}$ residues remained small throughout (0.23 Å). However, the absence of CaV$_{\beta}$-binding and modulation with the I14D mutant is likely to stem from the relatively higher electrostatic energy produced by the net negative charge and the lower hydrophobicity of the aspartate residue as compared with the arginine residue. The three-dimensional model also infers that the aspartate residue could not form additional hydrogen bonds unlike the arginine residue. Although these bonds are non-existent with the native isoleucine, they could have minimized the energy of the CaV$_{\beta}$-CaV$_{AID}$ complex. The negative charge of I14D would then be associated with a high energetic cost of burial in the hydrophobic environment of the binding pocket (42, 43). These properties could become noteworthy considering the hydrophobic character of the protein-protein interface between the CaV$_{\alpha 1}$ and the CaV$_{\beta}$ subunits.

Since the binding pocket formed by Tyr$_{10}$ appears to be deeper than the Ile$_{14}$ site, bulky residues are expected to be better tolerated at Tyr$_{10}$ than at Ile$_{14}$. The substitution of the tyrosine by a phenylalanine at position 10 results in the loss of two hydrogen bonds with the CaV$_{\beta 2a}$ residues Ser$^{244}$ and Glu$^{305}$. Nonetheless, the aromatic rings of the tyrosine and the phenylalanine residues remain in the same orientation and the carbon atoms of the side-chains for both residues are in the same plane. The r.m.s.d. value for the AID$_h$ helix (0.18 Å) remained small suggesting that the Y10F mutation should not affect the CaV$_{\beta}$-CaV$_{AID}$ interaction as we have observed experimentally. Given that the binding pocket at this position is relatively deeper than the one at position 14, the arginine residue could eventually adopt an energetically favorable elongated configuration where its carbon atoms ($\alpha$, $\beta$, and $\gamma$) could espouse the same conformation as the equivalent carbon atoms from the native tyrosine residue. Indeed, the arginine residue appears to be as comfortably buried within CaV$_{\beta 2a}$ as the phenylalanine or the native tyrosine residues, which is substantiated by the small r.m.s.d. value observed with this mutant (0.23 Å). Finally, the arginine residue forms also a hydrogen bond with CaV$_{\beta 2a}$ like the native wild-type tyrosine residue does, albeit with a different residue (Asp$^{305}$). All these factors could account for the observation that the Y383R mutation did not affect significantly CaV$_{\beta}$ binding or CaV$_{\beta}$-induced modulation. In contrast, the aspartate residue at this position (Y10D) was too short to fully interact with CaV$_{\beta 2a}$ or to form any hydrogen bond with CaV$_{\beta 2a}$ residues. The shorter side-chain of the aspartate residue does not however appear to significantly affect the conformation of the AID$_h$ peptide since the r.m.s.d. value (0.24 Å) remained comparable to the values obtained for the arginine and the phenylalanine at this position. Hence the overall lower hydrophobicity as well as the absence of the hydrogen bond could explain in part the lack of CaV$_{\beta}$-induced modulation of the Y383D mutant in CaV$_2.3$.

CONCLUSION

Our data proved that mutations of residues located on the C-terminus of the AID helix (Glu$^6$, Leu$^5$) impact less significantly on CaV$_{\alpha}$ channel modulation by CaV$_{\beta}$ than mutations on the C-terminal end of the AID helix (Trp$^{13}$, Ile$^{14}$). These data agree with the three-dimensional model of CaV$_{2.3}$/CaV$_{\beta 2a}$ built using the crystal coordinates from CaV$_{1.2}$/CaV$_{\beta 2a}$. However, CaV$_{\beta}$ binding to the denatured AID peptide did not appear to completely account for the interaction between the CaV$_{\alpha 1}$ and the CaV$_{\beta}$ subunits. In particular, CaV$_{\beta}$ binding to the AID peptide was ablated after conserved and nonconserved mu-
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The C-terminal Residues in the Alpha-interacting Domain (AID) Helix Anchor CaVβ Subunit Interaction and Modulation of CaV2.3 Channels
Laurent Berrou, Yolaine Dodier, Alexandra Raybaud, Audrey Tousignant, Omar Dafi, Joelle N. Pelletier and Lucie Parent

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