Mutations of Nonconserved Residues within the Calcium Channel α₁-interaction Domain Inhibit β-Subunit Potentiation

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Voltage-dependent calcium channels consist of a pore-forming subunit (CaVα₁) that includes all the molecular determinants of a voltage-gated channel, and several accessory subunits. The ancillary β-subunit (CaVβ) is a potent activator of voltage-dependent calcium channels, but the mechanisms and structural bases of this regulation remain elusive. CaVβ binds reversibly to a conserved consensus sequence in CaVα₁, the α₁-interaction domain (AID), which forms an α-helix when complexed with CaVβ. Conserved aromatic residues face to one side of the helix and strongly interact with a hydrophobic pocket on CaVβ. Here, we studied the effect of mutating residues located opposite to the AID-CaVβ contact surface in CaV1.2. Substitution of AID-exposed residues by the corresponding amino acids present in other CaVα₁ subunits (E462K, K465N, D469S, and Q473K) hinders CaVβ’s ability to increase ionic-current to charge-movement ratio (I/Q) without changing the apparent affinity for CaVα₁. At the single channel level, these CaV1.2 mutants coexpressed with CaVβ₃ visit high open probability mode less frequently than wild-type channels. On the other hand, CaV1.2 carrying either a mutation in the conserved tryptophan residue (W470S, which impairs CaVβ binding), or a deletion of the whole AID sequence, does not exhibit CaVβ-induced increase in I/Q. In addition, we observed a shift in the voltage dependence of activation by +12 mV in the AID-deleted channel in the absence of CaVβ, suggesting a direct participation of these residues in the modulation of channel activation. Our results show that CaVβ-dependent potentiation arises primarily from changes in the modulating gating behavior. We envision that CaVβ spatially reorients AID residues that influence the channel gate. These findings provide a new framework for understanding modulation of VDCC gating by CaVβ.

INTRODUCTION

Influx of calcium from the extracellular medium is mainly mediated by voltage-dependent calcium channels, which are classified according to their threshold into high or low voltage of activation (Ertel et al., 2000). Channels from the high voltage of activation family are composed of at least four nonhomologous subunits: the main pore-forming subunit, CaVα₁, and three auxiliary subunits, CaVα₂/B, CaVβ, and CaVγ. CaVα₁ encodes all the structural elements of a functional voltage-activated calcium channel (Catterall, 2000). Among the auxiliary subunits, CaVβ stands out as the most potent regulator of channel function and expression (Hidalgo and Neely, 2007; Dolphin, 2003). Four CaVβ isoforms (CaVβ₁ to CaVβ₄) have been cloned from four nonallelic genes, each encoding multiple splice variants. Although all CaVβ isoforms potentiate currents mediated by any of the CaVα₁-encoding high voltage of activation channels, they do so to a different extent (Dzhura and Neely, 2003; Luvisetto et al., 2004). This current potentiation manifests itself in an increase in the ionic-current to charge-movement ratio (I/Q) (Neely et al., 1993; Olcese et al., 1996) and in the channel’s open probability (Po) (Wakamori et al., 1993; Neely et al., 1995; Costantin et al., 1998; Wakamori et al., 1999; Dzhura and Neely, 2003). CaVβ may also increase the number of channels present in the plasma membrane by releasing them from the endoplasmic reticulum (Bichet et al., 2000). All CaVβ isoforms are also capable of modulating inactivation, but only CaVβ₂a inhibits voltage-dependent inactivation whereas CaVβ₁b, CaVβ₃, and CaVβ₄ promotes it (Olcese et al., 1994; Qin et al., 1996; Sokolov et al., 2000; Restituito et al., 2000; Hering et al., 2000). A subset of CaVβ isoforms participate as well in the regulation of calcium channel by G-proteins (Sandoz et al., 2004a) or protein phosphorylation (Arikkath and Campbell, 2003). This diversity in modulatory capabilities stands in contrast with the existence of a single well-defined binding site shared by all CaVα₁ subunits. In CaVα₁, this interaction involves a highly conserved consensus sequence, the α₁-subunit...
interaction domain (AID), which lies within the cytoplasmic loop joining the first and second repeat of Ca\(\alpha_1\) (Pragnell et al., 1994; Fig. 1 A). According to the crystal structure of the AID–Ca\(\beta\) complex, AID adopts an \(\alpha\)-helical structure with the fully conserved tyrosine and tryptophan residues lying on one face of the helix, and becoming buried in a hydrophobic pocket within Ca\(\beta\), the so called \(\alpha\)-binding pocket (Opatowsky et al., 2004; Van Petegem et al., 2004; Chen et al., 2004). Mutations in the AID sequence change inactivation of Ca\(\alpha_1\) (Leroy et al., 2005; Berrou et al., 2001, 2002; Geib et al., 2002; Dafi et al., 2004), suggesting that this region interacts directly with a yet to be identified component of the channel gating machinery. Residues lying on the opposite face of the AID–Ca\(\beta\) interaction surface (AID-exposed residues, Fig. 1 A) would be available for this interaction. Interestingly, these residues are poorly conserved among Ca\(\alpha_1\) isoforms and may be partly responsible for isoform-specific variations. Because Ca\(\beta\)’s effect on channel activation is independent of its impact on inactivation (Olcese et al., 1994), we investigated the role of the AID motif in Ca\(\beta\)-dependent potentiation by either deleting it or replacing conserved and nonconserved amino acids within it. We chose Ca\(\alpha_1.2\) for it shows the largest shift in voltage activation by Ca\(\beta\) (Olcese et al., 1996). In the absence of Ca\(\beta\), mutations of AID residues (E462R, K465N, D469S, and Q473K) did not alter the voltage dependence of activation, I/Q, or single channel kinetics. In contrast, Ca\(\beta\)-induced increases in I/Q and Po were severely reduced in Ca\(\alpha_1.2\) channels bearing mutations in AID-exposed residues while the apparent binding affinity for Ca\(\beta\) remained unchanged. These mutant channels visit high Po mode less frequently than do wild-type (WT) channels without causing obvious changes in the kinetic within each gating mode. Deletion of the complete AID sequence (Ca\(\alpha_1.2\ ΔAID\)) shifts the voltage dependence of activation by +12 mV, suggesting that AID residues by themselves partially display the ability to modulate activation. Collectively, these findings indicate that in Ca\(\alpha_1.2\), AID-exposed residues interact with the gating machinery controlling modal gating once they are properly oriented upon Ca\(\beta\) binding.

**MATERIALS AND METHODS**

**Mutagenesis**

Amino acid substitutions were performed by overlapping PCR using two complementary oligonucleotides bearing the appropriate point mutation and two flanking oligonucleotides to amplify a 388-bp segment flanked by BamHI and SpeI restriction sites. The PCR product was digested with BamHI and SpeI and subcloned into pAGA2 vector carrying the coding sequence for Ca\(\alpha_1.2\) (GenBank accession no.: X15539). The SpeI silent site was incorporated by standard PCR methods in the Ca\(\alpha_1.2\) sequence at position 497. Constructs were selected by restriction pattern and confirmed by automated DNA sequencing. Ca\(\alpha_1.2\ ΔAID\) was constructed by removing residues 459 to 475 (459-QLEEDLKYLGDWITQAE-475). In all experiments, a variant of Ca\(\alpha_1.2\) bearing a deletion of 60 amino acids at the N terminus that increases expression was used (Wei et al., 1996).

**Electrophysiological Recordings and Oocyte Injection**

Capped cRNAs were synthesized from Hind III-linearized templates using the MESSAGE machine (Ambion), as described previously (Hidalgo et al., 2006). Proteins and cRNAs were injected into Xenopus oocytes using a nanoliter injector (Nanoliter 2000; World Precision Instruments). Ca\(\beta_2\alpha\) protein was purified and handled as described by Hidalgo et al. (2006). Electrophysiological recordings were performed as in Dzhura and Neely (2003). Macroscopic currents were recorded using the cut-open oocyte voltage-clamp technique (Taglialatela et al., 1992) with a CA-1B amplifier (Dagan Corporation) 4–5 d after cRNA injection and 2–5 h after protein injection, as described in Gonzalez-Gutierrez et al. (2007). The external solution contained 10 mM Ba\(^{2+}\), 96 mM n-methylglucamine, and 10 mM HEPES, and was adjusted to pH 7.0 with methanesulfonic acid. The internal solution contained 120 mM n-methylglucamine, 10 mM EGTA, and 10 mM HEPES, pH 7.0, adjusted with methanesulfonic acid. Pipettes were filled with 2 M tetramethylammonium-methanesulfonate, 50 mM NaCl, and 10 mM EGTA and showed a resistance from 0.5 to 1.2 M\(\Omega\). Current recordings were filtered at 10 kHz, and the linear components were subtracted by a P/−4 prepulse protocol. For patch-clamp recordings of single channel activity, we used an Axopatch-200B (MDS Analytical Technologies). Patch pipettes were pulled from aluminum silicate capillary (Sutter Instrument) coated with Silgard 184 (Dow Corning Corporation) and filled with a solution containing 76 mM Ba\(^{2+}\), 10 mM HEPES, and 100 mM S-cysteine (Sigma-Aldrich). It was adjusted to pH 7.0 with methanesulfonic acid. Pipette resistance ranged from 3 to 8 M\(\Omega\). Oocytes were placed in the recording chamber containing 110 mM K\(^+\) and 10 mM HEPES titrated to pH 7.0 with methanesulfonic acid. Calcium channels were activated by 200-ms pulses to 0 mV at
dependence of activation in Ca_{1.2} ΔAID channels appears shifted 12 mV toward more positive potentials (Fig. 2 B and Table S2). Half-voltages \( (V_{1/2} \) for both Boltzmann components describing the conductance versus voltage plot were shifted to the right by \(~10\) mV, and this difference was statistically significant (\( P < 0.01; \ t \) test). This suggests that residues within the AID sequence participate in Ca_{\beta}-mediated regulation of the coupling between voltage sensor and the channel gate.

Mutations of AID-exposed Residues Inhibit Ca_{\beta}-mediated Increase in I/Q.

To further investigate the role of AID-exposed residues in channel modulation, we replaced them by the corresponding amino acids present in other Ca_{\alpha}\textsubscript{1} subunits (Fig. 1) to yield Ca_{1.2} E462R, Ca_{1.2} K465N, Ca_{1.2} D469S, and Ca_{1.2} Q473K. We also tested the effect of a charge conservative mutation by replacing glutamate at position 462 by aspartate (Ca_{1.2} E462D). Fig. 3 A shows voltage-clamp recordings from Ca_{1.2} E462D, Ca_{1.2} W470S, and Ca_{1.2} D469S.

Online Supplemental Material

The online supplemental material includes four tables and six figures. Table S1 summarizes maximal I/Q values. Table S2 describes the parameters defining the sum of two Boltzmann distributions that best fits the normalized conductance curve (GV) for mutant and WT channels in the absence or presence of Ca_{\beta}_{2a}. Table S3 includes parameters defining the exponential distribution that best fits shut, open, and burst-duration histograms for mutants and WT channels with Ca_{\beta}. Table S4 contains the parameters defining the exponential that best fits burst histograms from individual patches of WT and E462R and K465N mutants of Ca_{1.2} without Ca_{\beta}_{2a}. Fig. S1 compares ionic currents and 1/Q versus voltage plot of Ca_{1.2} WT, Ca_{1.2} D469S, and Ca_{1.2} ΔAID in the presence or absence of Ca_{\beta}_{2a}. Fig. S2 compares 1/Q versus voltage plots for Ca_{1.2} WT, Ca_{1.2} E462R, Ca_{1.2} K465N with Ca_{\beta}_{2a}, or Ca_{\beta}_{2a}. Fig. S3 shows single channel current versus voltage plot for Ca_{1.2} WT, Ca_{1.2} D469S, Ca_{1.2} Q473K, and Ca_{1.2} ΔAID coexpressed with Ca_{\beta}_{2a}. Examples of single channel activity from Ca_{1.2} ΔAID and Ca_{1.2} WT are shown in Fig. S4. Dwell-time and burst-duraction histograms are plotted in Figs. S5 and S6, respectively. The online supplemental material is available at http://www.jgp.org/cgi/content/full/jgp.2007099901/DC1.

RESULTS

Deletion of AID Yields Ca_{\beta}-insensitive Channels and Induces a Right Shift in the Activation Curve

Mutating the conserved tryptophan residue within the AID (Ca_{1.2} W470S described in Hidalgo et al., 2006) or deleting the AID sequence (as in Gonzalez-Gutierrez et al. [2007]) of Ca_{1.2} does not affect channel expression but abolishes Ca_{\beta}-induced increase in I/Q (Fig. S1 and Table S1, available at http://www.jgp.org/cgi/content/full/jgp.2007099901/DC1). Fig. 2 B shows that there is a near perfect overlap in the activation curves of Ca_{1.2} W470S with Ca_{1.2} WT, independently of whether the mutant channel was expressed alone or together with Ca_{\beta}_{2a}. Here, we report that in addition, the voltage
E462R, and Ca\textsubscript{v}1.2 K465N. In the absence of Ca\textsubscript{v}\beta\textsubscript{2a}, all mutants yielded I/Q versus voltage curves (Fig. 3 B) and peak values (Table S1) that were indistinguishable from Ca\textsubscript{v}1.2 WT. Except for E462D, there were no apparent differences in the kinetic or voltage dependence of macroscopic currents recorded from oocytes expressing these variants in the presence of Ca\textsubscript{v}\beta\textsubscript{2a}. However, closer inspection revealed that inward ionic currents appeared reduced relative to the transient outward current (gating currents) for both mutants carrying a charge modification. Peak I/Q, when Ca\textsubscript{v}\beta\textsubscript{2a} was coexpressed, for Ca\textsubscript{v}1.2 E462R and Ca\textsubscript{v}1.2 K465N were 23 and 31% of Ca\textsubscript{v}1.2 WT, respectively, whereas Ca\textsubscript{v}1.2 E462D in the presence or absence of Ca\textsubscript{v}\beta was nearly identical to Ca\textsubscript{v}1.2 WT (Fig. 3, B and C, and Table S1). Thus, Ca\textsubscript{v}\beta\textsubscript{2a} increases peak I/Q of Ca\textsubscript{v}1.2 WT over 20-fold compared with sixfold and threefold for Ca\textsubscript{v}1.2 E462R and Ca\textsubscript{v}1.2 K465N channels, respectively. The other two mutations examined (D469S and Q473K), when coexpressed with Ca\textsubscript{v}\beta\textsubscript{2a}, also yield a peak I/Q smaller than Ca\textsubscript{v}1.2 WT (Table S1).

To test whether these mutations hindered potentiation by other Ca\textsubscript{v}\beta isoforms, we coexpressed the isoform endogenous to \textit{Xenopus} oocytes, Ca\textsubscript{v}\beta\textsubscript{3x} (Tareilus et al., 1997), and a rat neuronal isoform (Ca\textsubscript{v}\beta\textsubscript{1b}). Ca\textsubscript{v}\beta\textsubscript{3x} increased peak I/Q of WT Ca\textsubscript{v}1.2 channels by \sim 12-fold, whereas Ca\textsubscript{v}\beta\textsubscript{1b} did so by 10-fold. More importantly, Ca\textsubscript{v}\beta\textsubscript{3x} and Ca\textsubscript{v}\beta\textsubscript{1b} potentiated Ca\textsubscript{v}1.2 E462R and K465N channels to a lesser extend than did WT channels (Table S1 and Fig. S2, available at http://www.jgp.org/cgi/content/full/jgp.200709901/DC1). These reductions in I/Q were not accompanied by changes in the voltage dependence of activation nor in the ability of Ca\textsubscript{v}\beta\textsubscript{2a} to shift the GV curves toward negative potentials (Fig. 4, A and B, and Table S2). However, the maximal conductance (G\textsubscript{max}) normalized by charge movement (Q\textsubscript{on}) was greatly reduced for E462R and K465N mutants in the presence of Ca\textsubscript{v}\beta\textsubscript{2a} (Fig. 4 C).

**Figure 3.** Mutations of AID-exposed residues reduce I/Q only in the presence of Ca\textsubscript{v}\beta\textsubscript{2a}. (A) Macroscopic currents from oocytes coexpressing Ca\textsubscript{v}\beta\textsubscript{2a} with Ca\textsubscript{v}1.2 E462D, Ca\textsubscript{v}1.2 E462R, or Ca\textsubscript{v}1.2 K465N during an IV stimulation protocol that consisted of 70-ms depolarizing pulses ranging from \(-50\) mV to \(+60\) mV in 10-mV increments from a holding voltage of \(-80\) mV. Calibration bars correspond to 20 ms and 200 nA. (B) Average I/Q versus voltage plots for the indicated Ca\textsubscript{v}1.2 subunits in the absence of Ca\textsubscript{v}\beta. (C) Same as B but in the presence of Ca\textsubscript{v}\beta\textsubscript{2a} subunit. For comparison, data from Ca\textsubscript{v}1.2 WT with and without Ca\textsubscript{v}\beta\textsubscript{2a} are shown as dashed lines in B and C.

**Figure 4.** Ca\textsubscript{v}\beta\textsubscript{2a} retains its ability to shift GV curves, but maximal conductances are reduced in channels bearing mutations of AID-exposed residues. (A) Macroscopic currents from oocytes coexpressing Ca\textsubscript{v}\beta\textsubscript{2a} with either Ca\textsubscript{v}1.2 E462R or Ca\textsubscript{v}1.2 K465N during the same stimulation protocol used in Fig. 2 A (shown at the top), with calibration bars corresponding to 20 ms and 200 nA. (B) GV curves in the presence (filled symbol) or absence (open symbol) of Ca\textsubscript{v}\beta\textsubscript{2a}. (C) Plots of tail current amplitudes normalized by Q\textsubscript{on} (I\textsubscript{tail}/Q\textsubscript{on}) for Ca\textsubscript{v}1.2 WT (■), Ca\textsubscript{v}1.2 E462R (●), and Ca\textsubscript{v}1.2 K465N (▲). I\textsubscript{tail}/Q\textsubscript{on} (mean ± SEM) versus voltage plots were fitted to the sum of two Boltzmann distributions. The maximal I\textsubscript{tail}/Q\textsubscript{on} was 17.8 ± 2.5 nA/pC (n = 24) for Ca\textsubscript{v}1.2 WT + Ca\textsubscript{v}\beta\textsubscript{2a}, 7.4 ± 1.74 nA/pC (n = 11) for Ca\textsubscript{v}1.2 K465N + Ca\textsubscript{v}\beta\textsubscript{2a}, and 3.5 ± 0.8 nA/pC (n = 10) for Ca\textsubscript{v}1.2 E462R + Ca\textsubscript{v}\beta\textsubscript{2a}. 

Inhibition of β-Subunit Potentiation of Calcium Channel
We examined four factors that may account for this reduction in I/Q and Gmax: (1) a shift in the equilibrium toward inactivated states, (2) a decrease in the fraction of channels bound to Caβ, (3) a decrease in unitary conductance, and (4) a reduction in the channel probability of being open.

**E462R or K465N Mutations Do Not Alter Channel Inactivation of Ca1.2 When Coexpressed with Caβ2a**

The participation of the I-II loop of Caα1i in the regulation of voltage-dependent inactivation is well documented (Stotz et al., 2004a,b; Sandoz et al., 2004b; Berrou et al., 2001). Moreover, WT Ca1.2 coexpressed with Caβ2a, and Caα1i undergoes an ultraslow inactivation that develops over several seconds (Ferreira et al., 2005). Here, in the absence of Caα1i, the fraction of channels that inactivated after a 10-s depolarizing pulse did not exceed 30% over a wide range of voltages. Fig. 5 A shows the time courses of inward currents during a single 10-s pulse to 0 mV. The percentage of residual current remaining at the end of the pulse was virtually identical for WT, E462R, and K465N Ca1.2 channels (Fig. 5 B). To evaluate whether the reduction in I/Q was due to an increase in the fraction of mutant channels remaining inactive, we compared currents recorded at 0 mV after a 10-s prepulse to either −120 or −60 mV (Fig. 5 C). We were unable to detect any differences; thus, the reduction in I/Q observed in the presence of Caβ2a cannot be attributed to an enhancement of a slow voltage-dependent inactivation.

**Figure 5.** Inactivation in the presence of Caβ2a is not altered by E462R or K465N mutations. (A) Macroscopic current traces of the indicated Ca1.2 variants during a 10-s depolarization pulse to 0 mV, with calibration bars corresponding to 5 s and 20 nA. (B) Bar plot of the percentage of currents remaining after a 10-s depolarization pulse to 0 mV for the Ca1.2 variants shown in A. Values, expressed as mean ± SEM, are 81 ± 3% (n = 7) for Ca1.2 WT, 80 ± 3% (n = 7) for Ca1.2 E462R, and 80 ± 2% (n = 8) for Ca1.2 K465N. (C) Superimposed macroscopic currents from the same oocytes shown in A during a 200-ms pulse to 0 mV after a 10-s prepulse to either −120 mV (black) or −60 mV (red), with calibration bars corresponding to 200 ms and 20 nA.

Ca1.2 WT, Ca1.2 E462R, and Ca1.2 K465N Channels Exhibit the Same Apparent Binding Affinity for Caβ2a

Because Caβ2a shifts normalized GV curves of mutant and WT channels to the same degree, a similar fraction of channels is expected to be complexed with the auxiliary subunit and, therefore, substitutions of AID-exposed residues should not influence the affinity of Caβ2a for the anchoring domain. Following the same strategy used previously (Hidalgo et al., 2006), we performed dose-response experiments for each variant of Ca1.2 with purified Caβ2a protein and estimated the fraction of bound channels by modeling I/Q versus voltage plots (Fig. 6, A and B). In the presence of Caβ2a, these plots differ in size and shape from the one recorded in oocytes expressing Ca1.2 variants alone. In oocytes exposed to intermediate concentrations of purified Caβ2a, I/Q plots could be described by the weighted sum of template I/Q curves from Ca1.2 alone and from Ca1.2-expressing oocytes injected with saturating concentration of Caβ2a. The relative weight of Ca1.2-β2a template was taken as the fraction of Caβ2a-bound channels (β2a-like). The apparent dissociation constants (Ks) were then calculated from the fit of β2a-like coefficients versus concentrations of purified Caβ2a plot to a standard Hill’s equation (Fig. 6 C). The resulting values for Ks’s were similar for all Ca1.2 variants (0.20 μM for WT Ca1.2, 0.22 μM for Ca1.2 E462R, and 0.25 μM Ca1.2 K465N). As in Hidalgo et al. (2006), the Hill coefficient was allowed to vary freely, and best fits were obtained with values between 1.4 and 1.6. As discussed in a previous paper (Hidalgo et al., 2006), this type of experiment does not rule out a second binding site, but this seems unlikely in light of experiments showing that channel modulation is fully recapitulated by covalently linking a single Caβ2a to Ca1.2 (Dalton et al., 2005). On the other hand, it is possible that full equilibrium was not reached when the experiments were performed. Nevertheless, the fact that Ks and Hill coefficient were virtually identical for all Ca1.2 variants strongly indicates that the fraction of Caβ2a-bound channels was similar in all cases, and that the substituted residues indeed did not influence AID–Caβ2a interaction. This result agrees with a recent report that Ks between Caβ and synthetic AID peptides derived from different Caα1i subtypes is nearly identical (Van Petegem et al., 2008).

**AID Mutations Reduce Po of Channels Coexpressed with Caβ**

To determine whether changes in unitary conductance contribute to the reduced I/Q in mutant channels, we measured single channel conductance in the presence of Caβ2a for all four mutants of AID-exposed residues (Ca1.2 E462R, Ca1.2 K465N, Ca1.2 D469S, and Ca1.2 Q473K) and found it to be ~17 pS for all them (Fig. S3, available at http://www.jgp.org/cgi/content/full/jgp.200709901/DC1). We then compared single...
channel activity in 1,000 traces that recorded the responses to 200-ms pulses to 0 mV repeated once a second as in Dzhura and Neely (2003). Fig. 7 A shows 20 consecutive traces with this protocol for Ca_{v}1.2 E462R and Ca_{v}1.2 K465N (data for Ca_{v}1.2 D469S and Ca_{v}1.2 Q473K is shown in Fig. S4). A decrease in the overall Po in all mutants is evident by simple inspection because of the prevalence of sweeps lacking channel openings. Channel activity in traces from K465N and E462R mutants (Fig. 7), and also from D469S and Q473K channels (Fig. S4), display long bursts. These were similar to the ones that dominated activity in Ca_{v}1.2 WT in the presence of Ca_{v}β_{2a}. In contrast, in Ca_{v}1.2 W470S, channel openings are rather brief, as reported for WT calcium channel expressed in the absence of Ca_{v}β (Dzhura and Neely, 2003). The Po for each trace, estimated as the fraction of time that the channel spends in the open state, was plotted with respect to trace number to yield a typical diary plot (Fig. 7 B). As reported for WT Ca_{v}1.2 (Costantin et al., 1998; Dzhura and Neely, 2003), these plots show that channel activity is clustered and all variants retain the ability to gate in the different modes. Each mode has its own set of transition rates that determine the channel Po within a trace, which is also revealed in Po histograms using log binning (Fig. 7 C). These histograms are clearly multimodal and demonstrate that the Po among active sweeps for Ca_{v}1.2 E462R and Ca_{v}1.2 WT were distributed similarly, whereas with Ca_{v}1.2 K465N, low Po sweeps were more frequent. However, low and high Po modes appear to peak at the same values for both mutants. This stands in clear contrast with Ca_{v}1.2 W470S, which is near mono-modal; it lacks sweeps with Po > 0.1, and it is dominated by sweeps with Po around 0.05. Although the chance of including...
quently (16.6 ± 2.5%) compared with 40.3 ± 12.0% for Ca V\textsubscript{1.2} K465N, 37.8 ± 13.8% for Ca V\textsubscript{1.2} D469S, and 41.9 ± 10.1% for Ca V\textsubscript{1.2} Q473K. It should be noted though, that K465N, D469S, and Q473K mutants display higher patch-to-patch variability in the modal gating behavior, with low Po traces ranging from 10 to 83%. All these changes contribute to a reduction in the overall Po, which, when measured as the average fraction of time spent in the open state, ranges from 0.21 ± 0.08 for Ca V\textsubscript{1.2} WT to values ranging from 0.05 to 0.03 for mutant channels.

To visualize the impact of the decrease in Po on the macroscopic currents, mean current traces from single channel recordings were built by averaging traces during 0 mV jumps, from multiple patches for several channel variants (Fig. 9). Ca V\textsubscript{1.2} WT yields 210 fA, which compares to 29 fA for E462R and 41 fA for K465N (Fig. 9 A). This corresponds to an 86 and 81% reduction for E462R and K465N, respectively, and nearly matches the reduction in I/Q and Po described above. Single channel activity was recorded in the presence of S(-)Bay K 8644. Because the modal gating behavior may be influenced by the presence of this agonist (Lacerda and Brown, 1989), we compared I/Q versus voltage curves in the presence of 0.1 μM S(-)Bay K 8644 and 76 mM external Ba\textsuperscript{2+} to mimic single channel recording conditions (Fig. 9 B).
I/Q's for Ca_{1.2} E462R and Ca_{1.2} K465N were 77 and 63% smaller than for Ca_{1.2} WT, respectively, under the same recording conditions. This compares to the 87 and 79% difference in I/Q's observed in 10 mM Ba^{2+} and in the absence of the calcium channel agonist, indicating that differences in Po cannot be attributed to a differential effect of S(-)Bay K 8644. Collectively, these data show that the reduction in current capacity in channels bearing a mutation in AID-exposed residues and in the presence of Ca\textsubscript{V}\textsubscript{1.2} results from a decrease in Po associated with a decrease in the time spent in high Po mode.

**Kinetics within Gating Modes** is similar in Channels Bearing Mutations of AID-exposed Residues and Coexpressed with Ca\textsubscript{V}\textsubscript{1.2}

To evaluate if AID-exposed residues also modulate gating within each mode, we performed a standard dwell-time analysis for the different mutants. Fig. 10 shows open and shut-time histograms from the same patches shown in Fig. 7, but displayed in Sine-Sigworth coordinates. The maximum likelihood algorithm (Sigworth and Sine, 1987) was used to optimize multi-exponential probability density functions. Histograms for Ca\textsubscript{V}\textsubscript{1.2} WT and mutant channels are better described by the sum of two exponential distributions. The mean lifetime of long- and short-lived open states are indistinguishable between Ca\textsubscript{V}\textsubscript{1.2} WT and channels bearing mutations of AID-exposed residues (Table S3, available at http://www.jgp.org/cgi/content/full/jgp.200709901/DC1). In contrast, single-channel activity from Ca\textsubscript{V}\textsubscript{1.2} W470S mimicked the
activity of WT channels recorded in the absence of CaVβ (Dzhura and Neely, 2003). The relative contribution of short-lived openings, typical of low Po activity, was significantly larger in mutants with 25% of the active traces being low Po (CaV1.2 K465N, CaV1.2 D469S, and CaV1.2 Q473K). This should not come as a surprise because the apparent mean open-time in low Po sweeps is shorter (Dzhura and Neely, 2003), and their contribution should be augmented in channels that visit this mode of gating more often. Differences in shut-interval histograms, meaning the lifetime and relative contribution of short and long events, were not correlated with the prevalence of the different gating mode in any obvious manner and did not help in detecting changes in the kinetic within modes.

We next compared burst-duration histograms containing all traces and described them by the sum of two exponential distributions (Fig. 11 A). In all cases, the fast component had a mean lifetime of a fraction of a millisecond, whereas longer-lived burst of openings had a mean duration around 10 ms for all variants, except CaV1.2 W470S. In this mutant, long-lived bursts averaged 4.0 ± 0.8 ms, similar to what was previously reported for WT channels lacking CaVβ (Dzhura and Neely, 2003). To separate bursts originating from different gating modes, histograms were built using traces with Po > 0.1 or 0 < Po ≤ 0.1. From this analysis, we can see that burst-duration histograms for Po ≤ 0.1 still required the sum of two exponential distributions for their description (Fig. 11 B), and that the time constants and relative contributions of both components were similar in all cases (Table S3). In contrast, burst-duration histograms from Po > 0.1 traces was described by a single exponential distribution with an estimated mean burst duration that ranges from 8.7 to 17.5 ms (Fig. 10 C, and Fig. S6 and Table S3, which are available at http://www.jgp.org/cgi/content/full/jgp.200709901/DC1). On average, mean burst duration for CaV1.2 WT is 11.9 ± 1.2 ms (n = 6), which compares to 10.9 ± 1.9 ms (n = 7) for CaV1.2 E462R. Although only one half of the patches displayed a reasonable number of traces with Po > 0.1, a similar value was obtained for K465N (10.9 ± 3.6 ms; n = 3). Similarly, mean burst durations obtained for CaV1.2 D469S and CaV1.2 Q473K were 9.1 ± 1.1 ms (n = 4) and 9.7 ± 3.2 ms (n = 4), respectively. Collectively, these results indicate that the kinetic within modes is rather insensitive to mutations of AID-exposed residues in contrast to the impact in modal gating.

Single Channel Behavior in the Absence of CaVβ Is Similar in WT CaV1.2, CaV1.2 E462R, and CaV1.2 K465N

In the absence of CaVβ, the different CaV1.2 mutants yielded similar I/Q’s (Fig. 3 B), suggesting that these AID mutations do not alter intrinsic gating of the calcium channel. As shown in Fig. 12, single channel activity appeared strikingly similar for all mutants. Unfortunately, channel activity underlying the expression of CaVα1 by itself is insufficient to rule out the presence of multiple channels; thus, long-shut intervals do not reflect a state lifetime. Consequently, only burst-duration histograms from all sweeps were compared, and two exponential distributions were required to describe them. All parameters for CaV1.2 E463R and CaV1.2 K465N turned out to be similar to those for WT channels (Table S4, available at http://www.jgp.org/cgi/content/full/jgp.200709901/DC1), indicating that the mutant phenotype is only expressed in the presence of CaVβ. This suggests that AID-exposed residues come into place to interact with the gating machinery only when they are bound to this auxiliary subunit.

DISCUSSION

The main conclusion of this work is that substitutions of residues on the opposite face of the AID–CaVβ
Inhibition of Cav_{1.2} Subunit Potentiation of Calcium Channel Function

When normalized by Q_{on}, demonstrates that channel function rather than trafficking or expression is altered by these mutations. In single channel recordings, interaction surface, the so-called AID-exposed residues, reduce channel current capacity only when Cav_β is present. The change in the ionic-current versus voltage relationship, when normalized by Q_{on}, demonstrates that channel function rather than trafficking or expression is altered by these mutations. In single channel recordings,

**Figure 11.** Burst-duration histograms for Cav_{1.2} WT, Cav_{1.2} E462R, Cav_{1.2} K465N, and Cav_{1.2} W470S in the presence of Cavβ_{2a}. Openings separated by brief closings of less than 1 ms were included in the same burst. To build histograms, bursts coming from all traces were used in A, traces with Po ≤ 0.1 were chosen in B, and traces with Po > 0.1 were used in C. In A and B, the sum of two exponential distributions was necessary to describe the data (red line). Individual exponential components are shown in blue and green. A single exponential distribution was sufficient to describe burst duration histogram from traces with Po > 0.1. For W470S, an exponential fit was not attempted due to the limited number of events. The same recordings shown in Fig. 7 were used here.

**Figure 12.** Single channel activity and burst duration histograms for Cav_{1.2} WT, Cav_{1.2} E462R, Cav_{1.2} K465N, and Cav_{1.2} W470S in the absence of Cavβ_{2a}. (A) Representative traces of patches containing Cav_{1.2} WT, Cav_{1.2} E462R, or Cav_{1.2} K465N in the absence of Cavβ_{2a} in identical recording condition as in Fig. 7. Calibration bar corresponds to 50 ms and 2 pA. (B) Burst-duration histograms for Cav_{1.2} WT, E462R, and K465N in the absence of Cavβ. As in Fig. 11, openings separated by brief closings of less than 1 ms were included in the same burst. All traces were used to build these histograms. A sum of two exponential distributions was necessary to describe the data.
there is dramatic reduction in the overall Po, whereas unitary conductance remains constant. As described for WT CaV1.2 (Dzhura and Neely, 2003), CaV1.2 E462R, CaV1.2 K465N, CaV1.2 D469S, and CaV1.2 Q473K present clusters of openings in at least two nonsilent modes that are manifested in Po histograms using logarithmic binning. However, the relative contribution of each nonsilent mode differs among these mutants. Whereas in CaV1.2 E462R the low Po mode is seldom visited, K465N, D469S, and Q473K mutant channels spend most of their time between low Po and silent modes. On the other hand, burst-duration histograms derived from sweeps with Po > 0.1 were similar for all mutants and WT channels, suggesting that in the presence of CaV\(_{\beta}\), AID-exposed residues modulate channel Po changes in modal gating rather than in the kinetic within mode.

Substitutions of the conserved tryptophan residue by serine (CaV1.2 W470S) yielded channels that behave as WT channels in the absence of CaV\(_{\beta}\) (Dzhura and Neely, 2003). This finding appears in discrepancy with previous results showing that CaV\(_{\beta}\)_2 modulator is spared when this conserved tryptophan residue is substituted by alanine in CaV2.2 channels (Leroy et al., 2005). Nevertheless, this tryptophan is buried in the \(\alpha\)-binding pocket of the \(\beta\)-subunit (Opatowsky et al., 2004; Van Petegem et al., 2004; Chen et al., 2004) and, according to a recent comprehensive survey on the energetic of CaV\(_{\alpha}\)–CaV\(_{\beta}\) interaction, this residue is critical for binding and modulation of function by CaV\(_{\beta}\) (Van Petegem et al., 2008).

There are several reports indicating that AID residues or more proximal elements of the I-II loop participate in channel inactivation (Sandoz et al., 2004b; Raybaud et al., 2007; Berrou et al., 2005; Cens et al., 2006). Furthermore, it has been reported that E462R mutant of CaV1.2 channels display an acceleration of voltage-dependent inactivation when combined to CaV\(_{\alpha}\)\(/\beta\) and CaV\(_{\beta}\)_3 (Berrou et al., 2001; Dafi et al., 2004). If the reduced Po that we observed on E462R plus CaV\(_{\beta}\)_3 stemmed from an increase in the inactivation rate, it would be visible in prolonged pulses, which we did not observe. Moreover, the fact that this mutation appears to impair CaV\(_{\beta}\)_3-mediated increase in I/Q to a larger extent than CaV\(_{\beta}\)_1b, which promotes inactivation, reinforces the idea that the effect of CaV\(_{\beta}\) on channel activation is independent of its impact on the rate of channel inactivation (Olcese et al., 1994).

Our data confirm that calcium channels coexpressed with CaV\(_{\beta}\) alternate between low Po and high Po modes (Shistik et al., 1995, Dzhura and Neely, 2003, Luvisetto et al., 2004). Because CaV\(_{\alpha}\)–CaV\(_{\beta}\) interaction is reversible (Hidalgo et al., 2006), it appears plausible that channels switch from low to high Po upon binding to CaV\(_{\beta}\). In such a case, a reduction in the fraction of time spent in the high Po mode should correlate with an increase in the proportion of channels lacking CaV\(_{\beta}\). Here, we show that CaV1.2 mutants that seldom visit the high Po mode maintain their apparent affinities constant for CaV\(_{\beta}\). This is entirely in line with experiments showing that AID derived from different calcium channel subtypes share the same affinity for CaV\(_{\beta}\) (Van Petegem et al., 2008). To explain changes in the prevalence among different gating modes, we envision instead that CaV\(_{\beta}\) remains attached to the channel and positions AID-exposed residues to interact with a still unknown region of CaV\(_{\alpha}\), and that this interaction may take two possible configurations to account for low and high Po modes. In the absence of this putative interaction, channel would remain in a silent mode. Changing the side chain of some of these residues, as in K465N, D469S, and Q473K mutants, destabilizes the configuration that supports high Po, and shifts the equilibrium toward low and silent mode, whereas channel-bearing E462R mutations spend most of their time in the silent mode because the stability of both configurations is reduced. Within this new framework, \(\beta\)-subunit potentiation of calcium channel would arise from an increase in the proportion of channels gating in the high Po mode, and the coupling efficiency between the voltage sensor and the channel can be regulated by changing the distribution of the different gating modes.

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