Cross talk between β subunits, intracellular Ca\textsuperscript{2+} signaling, and SNAREs in the modulation of Ca\textsubscript{V}2.1 channel steady-state inactivation

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Keywords  
Ca\textsuperscript{2+}-calmodulin, Ca\textsubscript{V}2.1 domains for SNARE-mediated modulation, Ca\textsubscript{V}2.1 steady-state inactivation, Ca\textsubscript{β} subunits, presynaptic voltage-gated Ca\textsubscript{V}2.1 channels, syntaxin-1A.

Abstract  
Modulation of Ca\textsubscript{V}2.1 channel activity plays a key role in interneuronal communication and synaptic plasticity. SNAREs interact with a specific synprint site at the second intracellular loop (LII-III) of the Ca\textsubscript{V}2.1 pore-forming α\textsubscript{1A} subunit to optimize neurotransmitter release from presynaptic terminals by allowing secretory vesicles docking near the Ca\textsuperscript{2+} entry pathway, and by modulating the voltage dependence of channel steady-state inactivation. Ca\textsuperscript{2+} influx through Ca\textsubscript{V}2.1 also promotes channel inactivation. This process seems to involve Ca\textsuperscript{2+}-calmodulin interaction with two adjacent sites in the α\textsubscript{1A} carboxyl tail (C-tail) (the IQ-like motif and the Calmodulin-Binding Domain (CBD) site), and contributes to long-term potentiation and spatial learning and memory. Besides, binding of regulatory β subunits to the α interaction domain (AID) at the first intracellular loop (LI-II) of α\textsubscript{1A} determines the degree of channel inactivation by both voltage and Ca\textsuperscript{2+}. Here, we explore the cross talk between β subunits, Ca\textsuperscript{2+}, and syntaxin-1A-modulated Ca\textsubscript{V}2.1 inactivation, highlighting the α\textsubscript{1A} domains involved in such process. β\textsubscript{3}-containing Ca\textsubscript{V}2.1 channels show syntaxin-1A-modulated but no Ca\textsuperscript{2+}-dependent steady-state inactivation. Conversely, β\textsubscript{2a}-containing Ca\textsubscript{V}2.1 channels show Ca\textsuperscript{2+}-dependent but not syntaxin-1A-modulated steady-state inactivation. A LI-II deletion confers Ca\textsuperscript{2+}-dependent inactivation and prevents modulation by syntaxin-1A in β\textsubscript{3}-containing Ca\textsubscript{V}2.1 channels. Mutation of the IQ-like motif, unlike CBD deletion, abolishes Ca\textsuperscript{2+}-dependent inactivation and confers modulation by syntaxin-1A in β\textsubscript{2a}-containing Ca\textsubscript{V}2.1 channels. Altogether, these results suggest that LI-II structural modifications determine the regulation of Ca\textsubscript{V}2.1 steady-state inactivation either by Ca\textsuperscript{2+} or by SNAREs but not by both.

Key points summary  
• The functional interaction between presynaptic voltage-gated Ca\textsuperscript{2+} channels (Ca\textsubscript{V}2.x) and soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins of the secretory machinery, optimizes neurotransmitter-mediated interneuronal communication.
• Alteration of such functional interaction has clinical relevance in the context of neurological disorders such as ataxia and migraine.
• Regardless of the important anchoring function of a specific CaV2.x region (the synprint site) in the CaV2.x-SNAREs interaction, the involvement of other channel domains has been proposed.

• By combining heterologous expression in HEK 293 cells, whole-cell patch-clamp and site-directed mutagenesis, we show that CaV2.1-SNAREs functional interaction entails CaV2.1 molecular determinants beyond the synprint site, including the first intracellular loop and the carboxyl tail, and their physical interaction with regulatory β subunits and the Ca\(^{2+}\)-calmodulin complex, respectively.

• Altogether help us better understand the molecular machinery that initiates and regulates vesicles fusion with the presynaptic plasma membrane to trigger chemical neurotransmission.

**Introduction**

Ca\(^{2+}\) entry through the high-voltage-activated (HVA) CaV2.x channels (mainly CaV2.1 [P/Q-type] channels) into presynaptic nerve terminals supports a transient Ca\(^{2+}\) microdomain that is essential for synaptic exocytosis leading to the fast release of classical neurotransmitters (Catterall 2011). To ensure fast and efficient neurotransmitter release, the vesicle-docking/release machinery must be located near the pathway of Ca\(^{2+}\) entry. In many cases, this close localization is achieved by direct interaction of soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins with the Ca\(^{2+}\) channel pore-forming σ1 subunit, which consists of four repeated domains (I-IV) each containing six transmembrane regions (S1–S6) with a voltage sensor (S1–S4) and a pore region (S5, P-loop, and S6). Indeed, syntaxin-1A/1B, SNAP-25, and synaptotagmin-1 specifically interact with CaV2.1 and CaV2.2 channels by binding to a synaptic protein interaction site (synprint) located within the intracellular loop connecting domains II and III (LII-III) of the channels (Sheng et al. 1994, 1997; Rettig et al. 1996; Kim and Catterall 1997; Jarvis et al. 2002) (Fig. 1A). Furthermore, it has been suggested that exocytosis is activated even before Ca\(^{2+}\) entry, by conformational changes triggered during Ca\(^{2+}\) binding at the open CaV channel pore, which are transmitted from the channel to specific residues of CaV-interacting SNARE proteins, accounting for the rapid time frame of evoked release (Atlas 2013; Bachnoff et al. 2013). Whichever the case, perhaps an equally important consequence of SNARE protein interaction with the Ca\(^{2+}\) channel is the modulation of presynaptic Ca\(^{2+}\) channel activity, thus fine-tuning the amount of Ca\(^{2+}\) that binds to the pore, enters the synaptic terminal, and determines synaptic transmission strength. Specifically, the binding of syntaxin-1A and SNAP-25 to CaV2.1 and CaV2.2 σ1 subunits shifts the voltage dependence of steady-state inactivation toward more negative membrane potentials following trains of brief depolarizing pulses to reduce channel availability, without affecting channel activation properties (Bezprozvanny et al. 1995; Zhong et al. 1999). Such inhibition is reverted, and channel activity fully restored, by synaptotagmin (Zhong et al. 1999). Thus, CaV2.x-SNAREs interaction seems to optimize neurotransmission by favoring Ca\(^{2+}\) entry through channels presenting docked synaptic vesicles. Accordingly, the disruption of such functional interaction compromises not just vesicle exocytosis in vitro (Mochida et al. 2003; Harkins et al. 2004), but also synaptic transmission and the SNARE-mediated inhibitory modulation of CaV2.x channels in vivo (Mochida et al. 1996; Rettig et al. 1997; Zamponi 2003; Keith et al. 2007). Moreover, human σ1A mutations impairing the functional interaction between CaV2.1 channels and SNARE proteins have clinical relevance in the context of ataxia and the phenotypic expression of both migraine with aura and hemiplegic migraine (Cricchi et al. 2007; Serra et al. 2010; Condille et al. 2013).

Beyond the important anchoring function of the synprint site in the SNARE-mediated modulation of CaV2.x channel gating, the involvement of other molecular domains has been proposed. Hence, deletions within the LII-III intracellular loop of the CaV2.2 σ1B channel subunit that completely eliminate the synprint site reduce but not abolish channel modulation by syntaxin, and syntaxin mutations that have no effect on binding affinity to σ1B-synprint prevent the SNARE-mediated regulation of CaV2.2 channel inactivation (Bezprozvanny et al. 2000). Besides, the A454T mutation (placed in the intracellular loop connecting domains I and II (LI-II) of the CaV2.1 σ1A channel subunit, and associated to both early-onset progressive ataxia (Cricchi et al. 2007) and the relief of migraine aura symptoms (Serra et al. 2010)) prevents the negative modulation of CaV2.1 channels by SNARE proteins and decreases channel coupling to exocytosis, thus revealing the importance of LI-II structural integrity in the CaV2.1-SNAREs functional interaction (Serra et al. 2010).

The molecular mechanism by which LI-II influences CaV2.1-SNAREs functional interaction is unknown. However, it is well established that LI-II plays a determinant role in the regulation of CaV2.1 channel activity (Buraei and Yang 2010). In this sense, it has been suggested that conformational changes induced at LI-II by the binding of functionally different regulatory β subunits not only determine the degree of voltage-dependent inactivation but also the extent of a Ca\(^{2+}\)-dependent inactivation component (mediated by the binding of Ca\(^{2+}\)-calmodulin to two adjacent sites in the carboxyl tail [C-tail] of the σ1A subunit: the IQ-like motif and the Calmodulin-Binding Domain [CBD] site) (Lee et al. 2000; DeMaria et al.
Interestingly, disruption of CaV2.1 modulation by calmodulin and related Ca²⁺ sensor proteins by mutation of the IQ-like motif has been reported to impair long-term potentiation and spatial learning and memory in mice (Nanoua et al. 2016). Altogether, it draws a complex scenario in which CaV2.1 inactivation is produced by LI-II and modulated by: β channel subunits interacting with LI-II, SNARE proteins binding to the synprint site at the LII-III but requiring the integrity of LI-II, and Ca²⁺-calmodulin attached to the C-tail of α₁A subunit.

To better understand the role of LI-II in CaV2.1-SNAREs functional interaction, we analyzed the modulation of CaV2.1 inactivation by syntaxin-1A under intermediate and high Ca²⁺-buffering conditions, in the presence of functionally different regulatory β subunits (β₂a or β₃) and distinct human α₁A constructs containing either a LI-II deletion around the A454 residue (ΔLI-II₄₅₁–₄₅₇) (also depicted at panel A).

Methods

cDNA constructs and site-directed mutagenesis
cDNA of the human voltage-gated Ca²⁺ (CaV2.1) channel α₁A subunit (originally cloned into a pCMV vector) was a gift from Professor J. Striessnig (University of Innsbruck, Austria). cDNAs of the rabbit α₂ and rat β₃ and β₂a regulatory subunits, and syntaxin-1A (subcloned into a pcDNA3 expression vector) were gifts from Dr. L. Birnbaumer (National Institutes of Health, North Carolina, USA) and Dr. J. Blasi (Universitat de Barcelona, Spain). CaV2.1 α₁A mutant subunits (ΔLI-IV₄₅₁–₄₅₇; IM/EE1964-1965, ΔCBD2020-end) were generated using site-directed mutagenesis (GenScript Corporation, Piscatway, NJ). All cDNA clones used in this study were sequenced in full to confirm their integrity.

Heterologous expression and electrophysiology

HEK 293 cells were transfected using a linear polyethyleneimine (PEI) derivative, the polycation ExGen500 (Fermentas Inc., Hanover, Maryland, USA) as previously
reported (eight equivalents PEI/3 μg DNA/dish) (Serra et al. 2010). For transfection, z1A (wild-type [WT] or mutants), β3 or β2, δ, and EGFP (transfection marker) cDNA constructs were used at a ratio of 1:1:1:0.3. When required, syntaxin-1A was also cotransfected at the same ratio as CaV2.1 channel subunit cDNAs. Electrophysiological recordings were obtained from EGFP-positive cells 24–48 h after transfection at room temperature (22–24°C).

Ca2+ currents (ICa2+) through WT or mutant CaV2.1 channels containing β3 or β2, regulatory subunits were recorded in the whole-cell configuration of the patch-clamp technique, using a D-6100 Darmstadt amplifier (List Medical, Germany). Pipettes had a resistance of 2–3 MΩ when filled with a solution containing (in mmol/L): 140 CsCl, 1 EGTA (intermediate Ca2+-buffering condition) or 10 BAPTA (high Ca2+-buffering condition), 4 Na2ATP, 0.1 Na3GTP, and 10 Hepes (pH 7.2–7.3 and 290–300 mOsmol/L). The external solution contained (in mmol/L): 140 tetraethylammonium-Cl (TEACl), 3 CsCl, 25 CaCl2, 1.2 MgCl2, 10 Hepes, and 10 D-glucose (pH 7.4 and 300–310 mOsmol/L). Previous work demonstrates that high levels of intracellular Ca2+ chelators (e.g., 10 mmol/L EGTA or 10 mmol/L BAPTA) impair CaV2.1 inactivation in a similar way as when replacing extracellular Ca2+ by Ba2+ (Lee et al. 2000). Such observation strongly suggest that chelator effect is due to Ca2+ buffering, ruling out any unwanted direct action of the Ca2+ chelator itself on channel inactivation. pClamp8 software (Molecular Devices, USA) was used for pulse generation, data acquisition, and subsequent analysis.

Steady-state inactivation was estimated by measuring peak Ca2+ currents in response to a 50 ms (or 10 ms, when using the z1A IM/EE mutant subunit) depolarizing test pulse (to +20 mV) from a holding of ~80 mV, following 30-sec steps to various holding potentials (conditioning pulses) between −80 and +20 mV (Fig. 1A). Between the 30-sec conditioning depolarizations and the test pulse we employed a 20-msec interpulse to the holding potential, which does not allow detectable recovery from inactivation of CaV2.x channels (Degtiar et al. 2000). This kind of protocol has been reported to detect significant increase in CaV2.x steady-state inactivation induced by syntaxin-1A (i.e., a left shift of V1/2 inact to more negative voltages by ~6 mV) (Degtiar et al. 2000). On the contrary, when using shorter (few seconds) conditioning pulses, the influence of syntaxin-1A on CaV2.x channel gating was barely detectable (Degtiar et al. 2000). These results are consistent with the action of SNAREs on slow rather than fast channel inactivation (Degtiar et al. 2000). As described in detail previously (Serra et al. 2010), normalized ICa2+-persistent currents were fitted to the following Boltzmann equation in order to obtain half-maximal voltage (V1/2 inact) and slope factor (k inact) for steady-state inactivation:

$$\frac{I}{I_{max}} = \frac{1}{1 + e^{\frac{V-V_{1/2\ inact}}{k_{inact}}}}$$

### Statistics

Data are presented as the means ± SEM, and n represents the number of cells recorded for each experimental condition. Statistical significance was tested using one-way Analysis of Variance (ANOVA) followed by a Bonferroni post hoc test. Differences were considered significant if P < 0.05. All statistical comparisons were performed using the GraphPad Instat software. All data are sample from Gaussian (normal) distributions (tested using the method Kolmogorov and Smirnov).

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**Figure 2.** Steady-state inactivation of CaV2.1 channels containing the regulatory β3 subunit is independent of intracellular Ca2+ signaling and is favored by syntaxin-1A (A) Voltage protocol for the study of CaV2.1 channels steady-state inactivation (see Methods for further details). Representative normalized Ca2+ current traces recorded at intermediate (1 mmol/L EGTA) (B) or high (10 mmol/L BAPTA) (C) intracellular Ca2+-buffering conditions from a HEK 293 cell expressing CaV2.1 channels composed of wild-type z1A, β2, δ, and EGFP (transfection marker) (conditioning pulses) between −80 and 0 mV in order to generate the corresponding mean steady-state inactivation curves (D), which were fitted to a single Boltzmann function (see Methods, eq. 1) to estimate the half-inactivation potentials (V1/2 inact) (E) for WT CaV2.1 channels containing the β3 subunit (WTβ3) in the absence (open circles) or presence (filled circles) of syntaxin-1A (stx 1A), at the above indicated intracellular Ca2+-buffering conditions. Average V1/2 inact and k inact values at intermediate Ca2+-buffering condition (1 mmol/L EGTA) were (in mV): WTβ3 (open circles, n = 18) −23.7 ± 0.83 and −5.16 ± 0.24; WTβ3 + stx 1A (filled circles, n = 12) −32.74 ± 1.46 and −5.5 ± 0.19, respectively. At high Ca2+-buffering condition (10 mmol/L BAPTA), average V1/2 inact and k inact values were (in mV): WTβ3 (open circles, n = 12) −20.74 ± 1.18 and −4.78 ± 0.16; WTβ3 + stx 1A (filled circles, n = 8) −25.68 ± 1.13 and −5.25 ± 0.21, respectively. a and b: P < 0.001 and P < 0.05 versus the corresponding control condition (absence of syntaxin-1A), respectively; c: P < 0.01 when compared to the intermediate Ca2+-buffering condition (1 mmol/L EGTA). No significant difference was found for k inact values (ANOVA P = 0.17).
Results

The impact of the SNARE protein syntaxin-1A on the steady-state inactivation of Ca\textsuperscript{2+} currents (I\textsubscript{ca}\textsuperscript{2+}) through wild-type (WT) Ca\textsubscript{v}2.1 channel containing the regulatory \(\beta_3\) subunit (WT\(\beta_3\)), was measured at intermediate (1 mmol/L EGTA) and high (10 mmol/L BAPTA) intracellular Ca\textsuperscript{2+}-buffering conditions to evaluate its calcium dependency. In both conditions, syntaxin-1A expression favored channel steady-state inactivation, as indicated by a significant left shift of \(V_{1/2}\text{inact}\) to more negative voltages (by ~5–9 mV) (Fig. 2B–E; Table 1). It must be noted that steady-state inactivation of WT\(\beta_3\) channels was poorly dependent on intracellular Ca\textsuperscript{2+} concentration, as no significant differences were found when comparing I\textsubscript{ca}\textsuperscript{2+} \(V_{1/2}\text{inact}\) values obtained at intermediate and high intracellular Ca\textsuperscript{2+}-buffering conditions in the absence of syntaxin-1A (Fig. 2B and C (left panels), D and E (open circles); Table 1).

Interestingly, the introduction of a small deletion around the A454 residue at the first intracellular loop of the pore-forming \(\alpha_{1A}\) subunit (ALI-II\textsubscript{A451–457}) (Fig. 1) made the steady-state inactivation of \(\beta_3\)-containing Ca\textsubscript{v}2.1 channels (ALI-II\(\beta_3\)) Ca\textsuperscript{2+}-dependent. On one hand, I\textsubscript{ca}\textsuperscript{2+} inactivation was reduced (with a significant ~8 mV right shift in the \(V_{1/2}\)inact) by increasing the buffering of intracellular Ca\textsuperscript{2+} (Fig. 3A and B (left panels), C and D (open circles); Table 1). On the other hand, such \(\alpha_{1A}\) LI-II deletion removed the modulatory action of syntaxin-1A on the steady-state inactivation of Ca\textsubscript{v}2.1 channels containing \(\beta_3\) at intermediate Ca\textsuperscript{2+}-buffering condition (Fig. 3A, C [left panel], and D; Table 1), when Ca\textsuperscript{2+} entry through the channel promotes inactivation. The effect of syntaxin-1A on the inactivation of the ALI-II\(\beta_3\) channel was recovered (\(V_{1/2}\)inact was significantly left-shifted by ~6 mV) by increasing intracellular Ca\textsuperscript{2+} buffering to abrogate the novel LI-II deletion-induced Ca\textsuperscript{2+}-dependent component of inactivation (Fig. 3B and C [right panel], D; Table 1).

As widely reported before (for a review see Buraei and Yang 2010), Ca\textsubscript{v}2.1 inactivation was substantially right-shifted to more depolarized potentials for \(\beta_2a\)-containing than for \(\beta_3\)-containing channels (Fig. 4 vs. Fig. 2; Table 1). Under this condition, unlike WT\(\beta_3\) channels, the steady-state inactivation of I\textsubscript{Ca}\textsuperscript{2+} through the \(\beta_{2a}\)-containing WT Ca\textsubscript{v}2.1 channel (WT\(\beta_2a\)) presented a Ca\textsuperscript{2+}-dependent component (Fig. 4) that is not affected by the deletion in the first intracellular loop of the \(\alpha_{1A}\) subunit (ALI-II\textsubscript{A451–457}) (Fig. 5). Thus, \(V_{1/2}\)inact was significantly shifted to less negative values for both WT\(\beta_2a\) and ALI-II\(\beta_2a\) channels (by ~9 mV) when increasing intracellular Ca\textsuperscript{2+} buffering (Fig. 4A and B (left panels), C and D (open circles); Fig. 5A and B (left panels), C and D (open circles); Table 1). Accordingly, such right shift in the voltage dependence of inactivation disappeared once the \(\beta_{2a}\)-containing Ca\textsubscript{v}2.1 channel was rendered insensitive to

| Ca\textsubscript{v}2.1 channel | 1 mmol/L EGTA | 10 mmol/L BAPTA |
|----------------|----------------|----------------
|                | \(\text{–stx }1\text{A}\) | \(\text{+stx }1\text{A}\) | \(\text{–stx }1\text{A}\) | \(\text{+stx }1\text{A}\) |
| WT\(\beta_3\)  | \(\text{–23.7 ± 0.83}\) | \(\text{–32.74 ± 1.46}\*} | \(\text{–20.74 ± 1.18}\) | \(\text{–25.68 ± 1.13}\) |
| \(\Delta\text{LI-II}\beta_3\) | \(\text{–29.58 ± 1.35}\) | \(\text{–30 ± 1.85}\) | \(\text{–21.84 ± 0.83}\*} | \(\text{–27.94 ± 1.15}\*} |
| WT\(\beta_{2a}\) | \(\text{–12.08 ± 1.36}\) | \(\text{–14.53 ± 1.02}\) | \(\text{–2.78 ± 0.95}\*} | \(\text{–7.57 ± 1.36}\*} |
| \(\Delta\text{LI-II}\beta_{2a}\) | \(\text{–11.47 ± 0.86}\) | \(\text{–13.57 ± 1.15}\) | \(\text{–2.58 ± 0.99}\*} | \(\text{–7.45 ± 1.15}\*} |
| IM/EE\(\beta_{2a}\) | \(\text{4.19 ± 0.8}\) | \(\text{–1.25 ± 1.71}\*} | \(\text{1.4 ± 0.95}\) | \(\text{–4.68 ± 1.71}\*} |
| \(\Delta\text{CBD}\beta_{2a}\) | \(\text{–12.48 ± 1.07}\) | \(\text{–14.75 ± 1.53}\) | \(\text{–4.43 ± 1.15}\**} | \(\text{–9.6 ± 1.61}\*} |

Data are presented as the means ± S.E.M, and \(n\) represents the number of cells recorded for each experimental condition. Red asterisks for significant increases (left shift of \(V_{1/2}\) inact to more negative voltages) in channel steady-state inactivation induced by syntaxin-1A versus the corresponding control condition (absence of syntaxin-1A). Blue asterisks for significant decreases (right shift of \(V_{1/2}\) inact to less negative voltages) in channel steady-state inactivation induced by high Ca\textsuperscript{2+} buffering when compared to the corresponding intermediate Ca\textsuperscript{2+}-buffering condition (1 mmol/L EGTA). (ANOVA followed by a Bonferroni post hoc test).

*\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\).
Figure 3. ΔLI-II at α1A LI-II promotes a Ca\(^{2+}\)-dependent component in the steady-state inactivation of Ca\(_{\nu}\)2.1 channels containing the auxiliary β3 subunit, and it prevents syntaxin-1A-mediated modulation. Typical normalized Ca\(^{2+}\) current traces recorded at intermediate (1 mmol/L EGTA) (A) or high (10 mmol/L BAPTA) (B) intracellular Ca\(^{2+}\)-buffering conditions from a HEK 293 cell expressing Ca\(_{\nu}\)2.1 channels composed of mutant ΔLI-II\(_{451-457}\) α\(_{1A}\) β\(_{3}\) and α\(_{2}\) subunits (ΔLI-II\(_{β3}\)) either in the absence (left) or presence (right) of syntaxin-1A (stx 1A).

Currents were elicited by 50-ms depolarizing steps to +20 mV applied after 30-sec depolarizing prepulses to the shown voltages. Corresponding mean normalized steady-state inactivation curves (C), and derived V\(_{1/2}\) inactivation (D) for ΔLI-II\(_{451-457}\) α\(_{1A}\) β\(_{3}\) mutant channels containing the β3 subunit (ΔLI-II\(_{β3}\)) in the absence (open circles) or presence (filled circles) of syntaxin-1A (stx 1A), at the above indicated intracellular Ca\(^{2+}\)-buffering conditions. Average V\(_{1/2}\) inact and k\(_{\text{inact}}\) values at intermediate Ca\(^{2+}\)-buffering condition (1 mmol/L EGTA) were (in mV): ΔLI-II\(_{β3}\) (open circles, n = 14) -29.58 ± 1.35 and -4.73 ± 0.16; ΔLI-II\(_{β3}\) + stx 1A (filled circles, n = 9) -30 ± 1.85 and -5.73 ± 0.35, respectively. At high Ca\(^{2+}\)-buffering condition (10 mmol/L BAPTA), average V\(_{1/2}\) inact and k\(_{\text{inact}}\) values were (in mV): ΔLI-II\(_{β3}\) (open circles, n = 9) -21.84 ± 0.83 and -4.94 ± 0.47; ΔLI-II\(_{β3}\) + stx 1A (filled circles, n = 8) -27.94 ± 1.15 and -4.97 ± 0.33, respectively. a: P < 0.001 when compared to the intermediate Ca\(^{2+}\)-buffering condition (1 mmol/L EGTA); b: P < 0.05 versus the corresponding control condition (absence of syntaxin-1A). No significant difference was found for k\(_{\text{inact}}\) values (ANOVA P = 0.14).
Ca\(^{2+}\) by the introduction of a double mutation (IM to EE) at the calmodulin-binding IQ-like motif (Fig. 6A and B [left panels], C and D [open circles]; Table 1). Modulation by syntaxin-1A of WT\(\beta_{2a}\) and \(\Delta LI-II/\beta_{2a}\) Ca\(^{2+}\) steady-state inactivation was occluded when the Ca\(^{2+}\)-dependent component was present (Fig. 4A and C [left panel], D; Fig. 5A and C [left panel], D; Table 1). Syntaxin-1A-induced left shift of \(V_{1/2}\) inact (by ~5–6 mV) was only present when the Ca\(^{2+}\)-dependent component of the steady-state inactivation of \(\beta_{2a}\)-containing CaV2.1 channels was removed, either by increasing intracellular Ca\(^{2+}\) buffering (Fig. 4B and C [right panel], D; Fig. 5B and C [right panel], D; Table 1) or by introducing the IM/EE double mutation at the IQ-like motif (Fig. 6A–D; Table 1).

Truncation of the \(\alpha_{1A}\) carboxyl tail, downstream the IQ-like motif, to fully remove the distal calmodulin-binding domain (CBD) site of the \(\beta_{2a}\)-containing CaV2.1 channel (\(\Delta CB\)D/\(\beta_{2a}\)) did not eliminate the Ca\(^{2+}\)-dependent component of \(I_{Ca}^{2+}\) steady-state inactivation, and \(V_{1/2}\) inact was still significantly shifted to less negative values (by ~9 mV) when increasing intracellular Ca\(^{2+}\) buffering (Fig. 7A and B [left panels], C and D [open circles]; Table 1). At intermediate intracellular Ca\(^{2+}\) buffering, the presence of the Ca\(^{2+}\)-dependent component in the steady-state inactivation of \(\Delta CB\)D/\(\beta_{2a}\) channels hindered their modulation by syntaxin-1A (Fig. 7A and C [left panel], D; Table 1), and the SNARE protein only shifted \(V_{1/2}\) inact to more negative potentials (by ~5 mV) under high intracellular Ca\(^{2+}\) buffering (Fig. 7B and C [right panel], D; Table 1).

**Discussion**

Taken together, our results bring to light a functional cross talk between three different signaling pathways regulating CaV2.1 channel steady-state inactivation: (1) regulatory \(\beta\) subunits through their interaction with the \(\alpha\) interaction domain (AID) located at the first intracellular loop (LI-II) of the CaV2.1 pore-forming \(\alpha_{1A}\) subunit (Buraei and Yang 2010), (2) Ca\(^{2+}\)-calmodulin binding to the IQ-like motif at the \(\alpha_{1A}\) C-tail (DeMaria et al. 2001; Cens et al. 2006; this report), and (3) syntaxin-1A, quite possibly, via its binding to the synprint site at the intracellular loop between domains II and III (LI-II-III) of \(\alpha_{1A}\) (Sheng et al. 1994, 1997; Rettig et al. 1996; Kim and Catterall 1997; Jarvis et al. 2002). As previously reported for fast inactivation (Lee et al. 2000), we observed a substantial Ca\(^{2+}\)-dependent component in the steady-state inactivation of CaV2.1 only in the presence of the palmitoylated, membrane-anchored \(\beta_{2a}\) subunit (which, contrary to other regulatory \(\beta\) subunits (such as \(\beta_{1}\) or \(\beta_{3}\)), reduces voltage-dependent inactivation (Birnbaumer et al. 1998). In agreement with findings from DeMaria et al. (2001) on CaV2.1 fast inactivation, the Ca\(^{2+}\)-dependent component of the steady-state slow inactivation required the Ca\(^{2+}\)-calmodulin-binding IQ-like motif, with no detectable role of the previously involved CBD site (Lee et al. 2000). Hence, CaV2.1 Ca\(^{2+}\)-dependent steady-state inactivation was abolished by the introduction of the double mutation IM/EE at the IQ-like motif, but unaffected by a truncation of the \(\alpha_{1A}\) C-tail, downstream the IQ-like motif, that fully removes the CBD site. Besides, the Ca\(^{2+}\)-dependent component of CaV2.1 steady-state inactivation seems to depend also on specific conformational changes induced by the binding of the functionally different \(\beta\) subunits at the \(\alpha_{1A}\) LI-II. Thus, the introduction of a LI-II deletion (\(\Delta LI-II_{520-532}\)) downstream the AID, around the A454 residue (of relevance for the modulation of CaV2.1 inactivation by \(\beta\) subunits and SNAREs (Serra et al. 2010)), made Ca\(^{2+}\)-sensitive the steady-state inactivation of \(\beta_{2a}\)-containing CaV2.1 channels. Such CaV2.1 LI-II deletion affects a fragment of a poorly conserved LI-II region of 13 amino acids that in the cardiac CaV1.2 channel can bind Ca\(^{2+}\)-calmodulin (Pitt et al. 2001) (Fig. 8). Still, removal of this CaV1.2 region (\(\Delta LI-II_{520-532}\)) did not abolish the Ca\(^{2+}\)-dependent component of cardiac channel inactivation (Pitt et al. 2001). This result agrees with our observation that \(\Delta LI-II_{451-457}\) had no effect on the Ca\(^{2+}\)-dependent inactivation of CaV2.1 channels containing the \(\beta_{2a}\) subunit.

Interestingly, syntaxin-1A was only able to modulate CaV2.1 steady-state inactivation when the Ca\(^{2+}\)-dependent component was absent either because of the presence of \(\beta_{3}\) in a channel formed by a \(\alpha_{1A}\) subunit with unaltered LI-II, or due to the removal of channel Ca\(^{2+}\)-sensitivity by high intracellular Ca\(^{2+}\) buffering or by mutation of the IQ-like motif.

Whether the above described functional cross talk is due to a three-dimensional rearrangement of the involved \(\alpha_{1A}\) intracellular domains (i.e., LI-II, LI-III and C-tail), and the subsequent alteration of the interaction pattern between them and/or with their interacting partners (regulatory \(\beta\) subunits, SNARE proteins, and the Ca\(^{2+}\)-calmodulin complex), remains to be elucidated. However, there is evidence that make this hypothesis plausible since it has been reported that N-tail, intracellular loop between domains III and IV (LI-IV) and C-tail regions of CaV2.2x or CaV1.2 \(\alpha\) subunits modulate channel inactivation through direct and dynamic interactions with LI-II, or indirectly via regulatory \(\beta\) subunits (Geib et al. 2002; Kim et al. 2004; Stotz et al. 2004). To date, there are no structural data regarding the whole CaV2.1 channel complex that allow us to confirm these physical interactions between \(\alpha_{1A}\) cytoplasmic domains. Nevertheless, the cryo-electron microscopy (cryo-EM) structure of the rabbit CaV1.1 complex, containing the pore-forming \(\alpha_{1S}\) and the...
Figure 4. Steady-state inactivation of CaV2.1 channels containing the regulatory β2a subunit presents a Ca²⁺-dependent component and no regulation by syntaxin-1A. Illustrative normalized Ca²⁺ current traces recorded at intermediate (1 mmol/L EGTA) (A) or high (10 mmol/L BAPTA) (B) intracellular Ca²⁺-buffering conditions from a HEK 293 cell expressing CaV2.1 channels composed of WT α₁a, β2a, and α₂δ subunits (WTβ2a), either in the absence (left) or presence (right) of syntaxin-1A (stx 1A). Currents were elicited by 50-ms depolarizing steps to +20 mV applied after 30-sec depolarizing prepulses to the indicated voltages. Corresponding mean normalized steady-state inactivation curves (C), and estimated $V_{1/2}$ inactivation (D) for ΔL-H551-457 CaV2.1 mutant channels containing the β2a subunit (WTβ2a) in the absence (open circles) or presence (filled circles) of syntaxin-1A (stx 1A), at the above indicated intracellular Ca²⁺-buffering conditions. Average $V_{1/2}$ inact and $k_{\text{inact}}$ values at intermediate Ca²⁺-buffering condition (1 mmol/L EGTA) were (in mV): WTβ2a (open circles, $n = 12$) −12.08 ± 1.36 and −2.43 ± 0.41; WTβ2a + stx 1A (filled circles, $n = 11$) −14.53 ± 1.02 and −2.33 ± 0.28, respectively. At high Ca²⁺-buffering condition (10 mmol/L BAPTA), average $V_{1/2}$ inact and $k_{\text{inact}}$ values were (in mV): WTβ2a (open circles, $n = 9$) −7.28 ± 0.95 and −3.1 ± 0.52; WTβ2a + stx 1A (filled circles, $n = 11$) −7.57 ± 1.36 and −3.15 ± 0.55, respectively. a: $P < 0.001$ when compared to the intermediate Ca²⁺-buffering condition (1 mmol/L EGTA); b: $P < 0.05$ versus the corresponding control condition (absence of syntaxin-1A). No significant difference was found for $k_{\text{inact}}$ values (ANOVA $P = 0.44$).
Steady-state inactivation of CaV2.1 channels formed by mutant α1AΔLI-II451–457 and β2a subunits remains Ca2+-dependent and syntaxin-1A-insensitive. Representative normalized Ca2+ current traces recorded at intermediate (1 mmol/L EGTA) (A) or high (10 mmol/L BAPTA) (B) intracellular Ca2+-buffering conditions from a HEK 293 cell expressing CaV2.1 channels composed of mutant ΔLI-II451–457α, β2a, and α2d subunits (ΔLI-IIβ2a) either in the absence (left) or presence (right) of syntaxin-1A (stx 1A). Currents were elicited by 50-ms depolarizing steps to +20 mV applied after 30-sec depolarizing prepulses to the indicated voltages. Amplitudes of currents elicited by test pulses to +20 mV after the different prepulses were normalized to the maximum current amplitude obtained after a 30-sec prepulse to /C0 80 mV in order to generate the corresponding mean steady-state inactivation curves (C), which were fitted to a single Boltzmann function (see Methods, eq. 1) to estimate the half-inactivation potentials (V 1/2 inactivation) (D) for mutant ΔLI-IIβ2a CaV2.1 mutant channels containing the β2a subunit (ΔLI-II/β2a) in the absence (open circles) or presence (filled circles) of syntaxin-1A (stx 1A), at the above indicated intracellular Ca2+-buffering conditions. Average V 1/2 inact and k inact values at intermediate Ca2+-buffering condition (1 mmol/L EGTA) were (in mV): ΔLI-IIβ2a (open circles, n = 8) −11.47 ± 0.86 and −2.87 ± 0.5; ΔLI-IIβ2a + stx 1A (filled circles, n = 9) −13.57 ± 1.15 and −2.73 ± 0.44, respectively. At high Ca2+-buffering condition (10 mmol/L BAPTA), average V 1/2 inact and k inact values were (in mV): ΔLI-IIβ2a (open circles, n = 9) −2.58 ± 0.99 and −2.05 ± 0.43; ΔLI-IIβ2a + stx 1A (filled circles, n = 9) −7.45 ± 1.15 and −2.12 ± 0.51, respectively. a: P < 0.001 when compared to the intermediate Ca2+-buffering condition (1 mmol/L EGTA); b: P < 0.01 versus the corresponding control condition (absence of syntaxin-1A). No significant difference was found for k inact values (ANOVA P = 0.51).
**Figure 6.** $\alpha_{1A}$ IQ-like motif mutation (IM/EE) remove the Ca$^{2+}$-dependent component in the steady-state inactivation of $\beta_{2a}$-containing Ca$\alpha_{2.1}$ channels, and it allows modulation by syntaxin-1A. Representative normalized Ca$^{2+}$ current traces recorded at intermediate (1 mmol/L EGTA) (A) or high (10 mmol/L BAPTA) (B) intracellular Ca$^{2+}$-buffering conditions from a HEK 293 cell expressing Ca$\alpha_{2.1}$ channels composed of mutant IM/EE $\alpha_{1A}, \beta_{2a}$, and $\alpha_{2d}$ subunits (IM/EE $\beta_{2a}$) either in the absence (left) or presence (right) of syntaxin-1A (stx 1A). Currents were elicited by 10-ms depolarizing steps to +20 mV applied after 30-sec depolarizing prepulses to the shown voltages. Corresponding mean normalized steady-state inactivation curves (C), and derived V$_{1/2}$ inactivation (D) for IM/EE Ca$\alpha_{2.1}$ mutant channels containing the $\beta_{2a}$ subunit (IM/EE $\beta_{2a}$) in the absence (open circles) or presence (filled circles) of syntaxin-1A (stx 1A), at the above indicated intracellular Ca$^{2+}$-buffering conditions. Average V$_{1/2}$ max and k$_{inact}$ values at intermediate Ca$^{2+}$-buffering condition (1 mmol/L EGTA) were (in mV): IM/EE $\beta_{2a}$ (open circles, $n = 10$) 4.19 ± 0.8 and -6.17 ± 0.5; IM/EE $\beta_{2a}$ + stx 1A (filled circles, $n = 11$) -1.25 ± 1.71 and -6.44 ± 0.34, respectively. At high Ca$^{2+}$-buffering condition (10 mmol/L BAPTA), average V$_{1/2}$ max and k$_{inact}$ values were (in mV): IM/EE $\beta_{2a}$ (open circles, $n = 7$) 1.4 ± 0.95 and -5.8 ± 0.62; IM/EE $\beta_{2a}$ + stx 1A (filled circles, $n = 9$) -4.68 ± 1.71 and -6.21 ± 0.42, respectively. a: $P < 0.05$ versus the corresponding control condition (absence of syntaxin-1A). No significant difference was found for k$_{inact}$ values (ANOVA $P = 0.82$).
Figure 7. Steady-state inactivation of CaV2.1 channels formed by mutant a1A, ΔCBD and β2a subunits still shows a Ca2+-dependent component and no regulation by syntaxin-1A. Typical normalized Ca2+ current traces recorded at intermediate (1 mmol/L EGTA) (A) or high (10 mmol/L BAPTA) (B) intracellular Ca2+-buffering conditions from a HEK 293 cell expressing CaV2.1 channels composed of mutant ΔCBD a1A, β2a and a2d subunits (ΔCBDβ2a) either in the absence (left) or presence (right) of syntaxin-1A (stx 1A). Currents were elicited by 50-ms depolarizing steps to +20 mV applied after 30-sec depolarizing prepulses to the shown voltages. Corresponding mean normalized steady-state inactivation curves (C), and derived V 1/2 inactivation (D) for ΔCBD CaV2.1 mutant channels containing the β2a subunit (ΔCBDβ2a) in the absence (open circles) or presence (filled circles) of syntaxin-1A (stx 1A), at the above indicated intracellular Ca2+-buffering conditions. Average V 1/2 inact and k inact values at intermediate Ca2+-buffering condition (1 mmol/L EGTA) were (in mV): ΔCBDβ2a (open circles, n = 8) -12.48 ± 1.07 and -2.23 ± 0.25; ΔCBDβ2a + stx 1A (filled circles, n = 6) -14.75 ± 1.53 and -2.59 ± 0.31, respectively. At high Ca2+-buffering condition (10 mmol/L BAPTA), average V 1/2 inact and k inact values were (in mV): ΔCBDβ2a (open circles, n = 12) -4.43 ± 1.15 and -3.68 ± 0.25; ΔCBDβ2a + stx 1A (filled circles, n = 12) -9.6 ± 1.61 and -4.52 ± 0.5, respectively. a: P < 0.01 when compared to the intermediate Ca2+-buffering condition (1 mmol/L EGTA); b: P < 0.05 versus the corresponding control condition (absence of syntaxin-1A). k inact values were significantly higher (P < 0.05) at high Ca2+-buffering condition (10 mmol/L BAPTA) than at intermediate Ca2+-buffering condition (1 mmol/L EGTA). The presence of syntaxin-1A had no significant effect on k inact.
Figure 8. Sequence alignment of intracellular loop between domains I and II (LI-II) of human CaV2.1 channel α1A subunit and rabbit CaV1.2 channel α1C subunit. Alignments were performed with Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). AID sequences are shown in purple, and rabbit (rb) α1C LI-II site for Ca2+-calmodulin binding is highlighted in green. Position of the human (h) α1A LI-II deletion around A454 (in red) (DLI-II451–457) is shown in orange. “*” identical residues; “:” conservative substitutions (same amino acid group); “.” semi-conservative substitution (similar shapes). LI-II residues appear in bold.

Figure 9. Sequence alignment of intracellular domains (LI-II, LIII-IV, and C-tail) of human CaV2.1 channel α1A subunit and rabbit CaV1.1 channel α1S subunit. Alignments were performed with Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). AID sequences at LI-II are shown in purple, and the human (h) α1A LI-II deletion around A454 (in red) (DLI-II451–457) is highlighted in orange. Amino acids involved in the physical interaction between LIII-IV and C-terminal domain (CTD) of rabbit (rb) α1S, according to cryo-EM structural data (Wu et al. 2016), and the homologous sequences in hα1A are shown in brown. Sequences of the IQ (rbα1S) and IQ-like (hα1A) motifs are shown in green. hα1A CBD sequence is depicted in blue. Cytoplasmic segments that were invisible in the cryo-EM structure of CaV1.1 rbα1S subunit (Wu et al. 2016) are shown in gray. “*” identical residues; “:” conservative substitutions (same amino acid group); “.” semi-conservative substitution (similar shapes). LI-II, LIII-IV, and CTD residues appear in bold.
CaV2.1 Sites for Functional Modulation by SNAREs

S. A. Serra et al.

regulatory $\alpha_1$, $\beta_1$, and $\gamma$ subunits, has been recently resolved with high, near-atomic (3.6Å) resolution (Wu et al. 2016). The structural analysis provides an atomic model for a potentially inactivated state of the CaV1.1 channel. In relation to the three-dimensional arrangement of $\alpha_1$ intracellular domains, the structural data locate the AID motif at LI-II packed in between the regulatory $\beta_1$ subunit and the voltage sensor of $\alpha_1$ domain II, and shows the formation of a globular helical domain due to the interaction between LIII-IV and the proximal C-tail (upstream the IQ motif) (Wu et al. 2016). The substantial homology between rabbit $\alpha_1$ and human $\alpha_1$ subunits with regard to residues involved in such LIII-IV/C-tail physical interaction (Fig. 9) suggests a similar scenario for the CaV2.1 channel. Unfortunately, several cytoplasmic segments were not visible in the cryo-EM structure of CaV1.1 $\alpha_1$ subunit, and the structure of LI-II upstream the AID, the whole LII-III and the C-tail after residue D1515 (including the IQ motif) could not be resolved (Wu et al. 2016). Therefore, there are no structural data available neither on the possible interaction of LI-II with either LIII-IV or the C-tail, or on any interaction involving LII-III.

Biochemical experiments with recombinant proteins in vitro strongly indicate that the synprint site, located at LII-III of $\alpha_1$ serves an important anchoring function that may facilitate SNARE’s modulation of CaV2.1 and CaV2.2 gating (Sheng et al. 1994, 1997; Rettig et al. 1996; Kim and Catterall 1997; Jarvis et al. 2002). Nonetheless, functional studies also suggest that the regulatory action of SNAREs might involve binding to other sites in the pore-forming $\alpha_1$ channel subunit, and LI-II and the C-tail regions have been proposed as candidates (Bezprozvanny et al. 2000; Serra et al. 2010). Supporting this idea, recent findings show that low voltage-activated CaV3.3 (T-type) $\alpha_1$ channel subunits, which do not contain the consensus synprint site, biochemically interact with syntaxin-1A and SNAP-25 at the carboxy-terminal domain (Weiss et al. 2012). In particular, syntaxin-1A binding to CaV3.3 channels potently modulates channel gating in a similar way that found for CaV2.2 channels (Weiss et al. 2012). Besides, CaV3.3-SNAREs interaction also appears essential for T-type channel-triggered low-threshold exocytosis (Weiss et al. 2012), thus providing a molecular mechanism for their coupling to neurotransmitter and hormone release in neurons and neuroendocrine cells near resting conditions or during mild stimulations (Carbone et al. 2014).

In conclusion, our data suggest that conformational modifications of $\alpha_1$ LI-II (due to the binding of a particular regulatory $\beta$ subunit, mutation A454T (Serra et al. 2010), or deletion ALI-II-L451–457) determine the modulation of CaV2.1 steady-state inactivation either by Ca$^{2+}$ or by SNAREs but not by both.

Acknowledgments

We are grateful to Dr. J. Striessnig (University of Innsbruck, Austria) for the gift of human CAGNA1A cDNA and Dr. J. Blasi (Universitat de Barcelona, Spain) for providing syntaxin-1A cDNA. We also thank Dr. L. Birnbaumer (National Institutes of Health, North Carolina, USA) for the gift of the cDNAs encoding rabbit $\alpha_1$, and rat $\beta_2$ and $\beta_3$ regulatory subunits, and Dr. F. Rubio-Moscó for excellent technical assistance.

Conflict of Interest

The authors declare that no conflict of interests exists.

In memoriam

In memory of Gemma G. Gen, PhD (1977–2017).

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