C-Terminal Modulatory Domain Controls Coupling of Voltage-Sensing to Pore Opening in Cav1.3 L-type Ca\(^{2+}\) Channels

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ABSTRACT Activity of voltage-gated Ca\(_{1.3}\) L-type Ca\(^{2+}\) channels is required for proper hearing as well as sinoatrial node and brain function. This critically depends on their negative activation voltage range, which is further fine-tuned by alternative splicing. Shorter variants miss a C-terminal regulatory domain (CTM), which allows them to activate at even more negative potentials than C-terminally long-splice variants. It is at present unclear whether this is due to an increased voltage sensitivity of the Ca\(_{1.3}\) voltage-sensing domain, or an enhanced coupling of voltage-sensor conformational changes to the subsequent opening of the activation gate. We studied the voltage-dependence of voltage-sensor charge movement (\(Q_{ON}\)) and of current activation (\(I_{Ca}\)) of the long (Ca\(_{1.3}\)) and a short Ca\(_{1.3}\) splice variant (Ca\(_{1.342A}\)) expressed in tsA-201 cells using whole cell patch-clamp. Charge movement (\(Q_{ON}\)) of Ca\(_{1.3}\) displayed a much steeper voltage-dependence and a more negative half-maximal activation voltage than Ca\(_{1.2}\) and Ca\(_{3.1}\). However, a significantly higher fraction of the total charge had to move for activation of Ca\(_{1.3}\) half-maximal conductance (Ca\(_{1.3}\): 68%; Ca\(_{1.2}\): 52%; Ca\(_{3.1}\): 22%). This indicated a weaker coupling of Ca\(_{1.3}\) voltage-sensor charge movement to pore opening. However, the coupling efficiency was strengthened in the absence of the CTM in Ca\(_{1.342A}\), thereby shifting \(I_{Ca}\) by 7.2 mV to potentials that were more negative without changing \(Q_{ON}\). We independently show that the presence of intracellular organic cations (such as \(n\)-methyl-D-glucamine) induces a pronounced negative shift of \(Q_{ON}\) and a more negative activation of \(I_{Ca}\) of all three channels. These findings illustrate that the voltage sensors of Ca\(_{1.3}\) channels respond more sensitively to depolarization than those of Ca\(_{1.2}\) or Ca\(_{3.1}\). Weak coupling of voltage sensing to pore opening is enhanced in the absence of the CTM, allowing short Ca\(_{1.342A}\) splice variants to activate at lower voltages without affecting \(Q_{ON}\).

INTRODUCTION

Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels (VGCCs) in the plasma membrane of electrically excitable cells causes membrane depolarization and triggers intracellular Ca\(^{2+}\)-dependent signaling processes. Ca\(_{1.3}\) and Ca\(_{1.2}\), members of the so-called L-type Ca\(^{2+}\) channel family (LTCCs, Ca\(_{1}\)), are widely expressed in many tissues, including muscle and neurons, sensory tissue, and endocrine cells (1–5). Work with genetically modified mice revealed different physiological roles for these two channel isoforms, even when expressed in the same cell (6–8). Differences in their voltage- and Ca\(^{2+}\)-dependent gating properties underlie this functional diversity (6–8). Ca\(_{1.3}\) channels activate faster and at more negative voltages than Ca\(_{1.2}\) (9–11) and therefore sustain Ca\(^{2+}\) inward currents also at threshold voltages. This allows them to control autonomous pacemaking in the sinoatrial node (2.6) and adrenal chromaffin cells (7) and support upstroke potentials in neurons (12). Ca\(_{1.3}\) channels are also expressed in the substantia nigra pars compacta dopaminergic neurons (13) where they seem to contribute to dendritic Ca\(^{2+}\) signals linked to mitochondrial stress and the selective vulnerability of these neurons in Parkinson’s disease (14). Selective Ca\(_{1.3}\) channel block is currently pursued as a therapeutic option for neuroprotection in Parkinson’s disease. Although Ca\(_{1.3}\) channels activate at voltages that are more negative than all other Ca\(_1\) (Ca\(_{1.1}\), Ca\(_{1.2}\), and Ca\(_{1.4}\)) and Ca\(_2\) high VGCCs (15), they cannot be classified as low VGCCs, such as T-type channels (16). Indirect comparisons of published data suggest that T-type channels activate and inactivate at voltages that are more negative than Ca\(_{1.3}\) (16), but a direct comparison of their gating properties, to our knowledge, does not exist.

Within the past few years, we have discovered that the activation voltage range (\(I_{Ca}\)) of Ca\(_{1.3}\) Ca\(^{2+}\) inward currents (\(I_{Ca}\)) can be shifted to potentials that are even more negative by alternative splicing within the C-terminus of its pore-forming \(\alpha_1\)-subunit (17.18). Alternative splicing generates Ca\(_{1.3}\) \(\alpha_1\)-subunits with shorter C-termini (e.g., Ca\(_{1.342A}\), Ca\(_{1.343S}\)) thereby removing a C-terminal modulatory domain (CTM) from long isoforms (Ca\(_{1.3}\)). This causes two apparently independent changes of channel gating:

1. It moderates Ca\(^{2+}\)-induced inactivation (CDI), an important autoinhibitory feedback mechanism of the channel. The molecular basis of this effect is well understood. As in other VGCCs, CDI is induced by Ca\(^{2+}\) binding to calmodulin (CaM) preassociated with the
proximal C-terminus of α1-subunits (21). The CTM of Ca\textsubscript{1.3} competitively interferes with CaM binding, and thereby reduces CDI (17,21).

2. The presence of the CTM in long Ca\textsubscript{1.3} splice variants reduces open probability at negative voltages and shifts the half-maximal activation voltage \(V_{0.5}(I_{Ca})\) by ~10 mV to potentials that are more positive. This modulation has been reported by different laboratories, and occurs independently of CaM (11,18,19).

In contrast to modulation of CDI, it remains unclear how the CTM can affect voltage-dependent gating. Contemporary homology models of the pore-forming α1-subunit propose four voltage-sensing domains (transmembrane segments S1–S4 of each of the four homologous repeats), which undergo conformational changes upon de- and repolarization of the channel. The intramembrane movement of the four positively charged S4 helices represents the main moving part (22,23). The cytoplasmic linkers between S4 and S5 segments are tightly packed into the pore-forming voltage-sensing machinery of all three VGCCs.

To address these questions, we established experimental conditions that allowed us to directly determine differences in gating properties of Ca\textsubscript{1.3} in comparison to Ca\textsubscript{1.2} and of short Ca\textsubscript{1.3} splice variants in comparison to long ones. We also systematically compared the gating properties of Ca\textsubscript{1.3} with low-voltage-activated Ca\textsubscript{1.3} T-type channels.

We found that Ca\textsubscript{1.3} \(Q_{ON}\) is significantly more voltage-sensitive than \(Q_{ON}\) of Ca\textsubscript{3.1} and Ca\textsubscript{1.2}. The coupling of voltage sensing to pore opening is less efficient in Ca\textsubscript{1.3} than in Ca\textsubscript{1.2}. The CTM did not affect the voltage-dependence of \(Q_{ON}\) of Ca\textsubscript{1.3}, indicating an inhibitory action on the transmission of voltage-sensor movements to pore opening. Surprisingly, intramembrane charge movement of Ca\textsubscript{1.3} occurred at voltages more positive than that of Ca\textsubscript{1.3}, but the sensitive coupling of the voltage sensors to pore opening accounted for low voltage activation of its \(I_{Ca}-V\). In the course of our work, we also discovered a strong effect of intracellular organic cations on the voltage-sensing machinery of all three VGCCs.

**METHODS**

**Cell culture and transient transfection**

HEK 293 (human embryonic kidney) cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 2 mM L-glutamine (Cat. No. 25030-032; Gibco, Life Technologies, Carlsbad, CA), 10 units/mL penicillin (Cat. No. P-3032; Sigma Aldrich, St. Louis, MO), 10 g/mL streptomycin (Cat. No. S-6501; Sigma), and with 10% v/v fetal calf serum (Cat. No. 10270-106; Gibco). Cells were grown under 5% CO\textsubscript{2} and 37°C until they reached 80% confluency. They were split with 0.05% trypsin for cell dissociation, and passage did not exceed 20. Transient transfection was achieved as described in Bock et al. (19), using equimolar cDNA ratios encoding Ca\textsubscript{1.3}, Ca\textsubscript{1.2}, or Ca\textsubscript{3.1} (generously provided by Norbert Klugbauer) together with auxiliary subunits β\textsubscript{1} and α\textsubscript{2}δ\textsubscript{1}. Cells were visualized by cotransfection of 1 μg GFP. Twenty-four hours after transfection, cells were plated on 35-mm polystyrene dishes, pretreated with 10 μg/mL poly-L-lysine. At 48–72 h after cell transfection, whole cell patch-clamp experiments were performed.

**Whole-cell patch-clamp recordings**

GFP-positive HEK 293 cells were recorded using the whole-cell patch-clamp configuration. Borosilicate glass electrodes, having a final resistance of 2–5 MΩ, were pulled with a micropipette puller (Sutter Instruments, Novato, CA) that was fire-polished (MF-830 microforge; Narishige, Tokyo, Japan). Data were digitized (Digitizer 1322A; Axon Instruments, Novato, CA) and recorded in the whole-cell patch-clamp configuration (Axopatch 200B; Axon Instruments).

**Intracellular recording solutions used**

- **NMDG<sub>int</sub>**: NMDG (150 mM n-methyl-D-glucamine), 10 mM EGTA, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, and 4 mM ATP-Mg, adjusted to pH 7.3 with MS (Methanesulfonate);
- **Cs<sub>nat</sub>**: 135 mM CsCl, 10 mM Cs-EGTA, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, and 4 mM ATP-Na\textsubscript{2}, adjusted to pH 7.3 with CsOH;
- **TRIS<sub>nat</sub>**: 164 mM Tris, 10 mM EGTA, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, and 4 mM ATP-Mg, adjusted to pH 7.3 with MS; and
- **TEA<sub>int</sub>**: 160 mM TEA (triethanolamine), 10 mM EGTA, 1 mM MgCl\textsubscript{2}, 10 mM HEPES, and 4 mM ATP-Mg, adjusted to pH 7.3 with MS.

**Extracellular solution used for \(Q_{ON}\) recordings**

Choline-Cl(\(\text{Mg}^{2+}\))\textsubscript{ext}: 150 mM choline-Cl, 16 mM MgCl\textsubscript{2}, 10 mM HEPES, 0.5 mM CdCl\textsubscript{2}, and 0.2 mM LaCl\textsubscript{3}, adjusted to pH 7.3 with CsOH.

**Extracellular solutions used for \(I_{Ca}\) recordings**

Choline-Cl\textsubscript{ext}: 150 mM choline-Cl, 15 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES, adjusted to pH 7.3 with CsOH;
- **Cs<sub>ext</sub>**: 150 mM CsCl, 15 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES, adjusted to pH 7.3 with CsOH; and
- **NMDG<sub>ext</sub>**: 146 mM NMDG, 15 mM CaCl\textsubscript{2}, 1 mM MgCl\textsubscript{2}, and 10 mM HEPES, adjusted to pH 7.3 with HCl.

Cells were maintained at a holding potential of ~80 mV, before a 25-ms- (\(I_{Ca}-V\)) or a 10-ms-long (\(Q_{ON}-V\)) square-pulse depolarization (2-s interpulse interval). P/Q leak subtraction was applied. \(Q_{ON}\) recordings were performed as described in Baig et al. (3).

To compare the \(Q_{ON}-V\) of Ca\textsubscript{1.3} and Ca\textsubscript{1.3}αδ, we quantified \(Q_{ON}\) \((\langle Q_{ON}\rangle)\) at \(V_{rev}\), after applying conditional prepulses in 5-mV steps to different potentials as previously described in McDonough et al. (24) and Baig et al. (3). During depolarizations to \(V_{rev}\), we measured the \(Q_{ON}\) not
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already moved during the prepulse. This allowed calculation of $Q_{\text{ON}}$-V from the remaining $Q_{\text{ON}}$ at $V_{\text{re}}$ after the indicated prepulses ($Q_{\text{ON, post-pre}}$). Using this protocol, we have previously also demonstrated that for Ca\textsubscript{v}1.3\textsubscript{L}, $Q_{\text{ON}}$-V is the same when Ca\textsuperscript{2+} in the extracellular solution is replaced by equimolar Mg\textsuperscript{2+} (+0.5 mM Cd\textsuperscript{2+} + 0.2 mM La\textsuperscript{3+}, see solutions above). This revealed no differences in surface charge effects and ruled out that the voltage-dependence of $Q_{\text{ON}}$ is affected by the Mg\textsuperscript{2+}-based extracellular solution used to block ionic current for $Q_{\text{ON}}$-measurements (3).

Steady-state activation (G-V) relationships were derived from $I_{\text{Ca}}$-V curves. We did not analyze tail current-voltage relationships due to contamination of tail currents by off-gating current in Cav1.2 and Cav1.3 channels. $I_{\text{Ca}}$-V of individual experiments was fitted to a modified Boltzmann equation,

$$ I = G_{\text{max}} \frac{(V - V_{\text{rev}})}{[1 + \exp\left(-\frac{(V - V_{0.5})}{k_{\text{act}}}\right)]}, $$

where $V_{\text{re}}$ is the extrapolated reversal potential, $V$ is the test potential, $I$ is the peak current amplitude, $G_{\text{max}}$ is the maximum slope conductance, $V_{0.5}$ is the half-maximal activation voltage, and $k_{\text{act}}$ is the slope factor. For fitting $I_{\text{Ca}}$-V curves recorded in the presence of intracellular organic cations (no current reversal observed due to the lack of outward current), data points at test pulses to voltages $>30$–$40$ mV were excluded from fitting. $Q_{\text{ON}}$-V and steady-state activation curves (G-V) were fitted to a Boltzmann equation,

$$ G(V) = \frac{G_{\text{max}}}{[1 + \exp(-\frac{V - V_{0.5}}{k_{\text{act}}})]}, $$

where $G_{\text{max}}$ is the saturating value and $k_{\text{act}}$ is the slope factor. Junction potentials were individually calculated for every solution combination, using the software included in the PCLAMP 10.2 software suite (Molecular Devices, Sunnyvale, CA), and offline-subtracted. (Missing ion mobility values in PCLAMP 10.2 were collected from http://web.med.unsw.edu.au/phbsoft/mobility_listings.htm (accessed November 27, 2013)).

**Junction potential corrections**

- $C_{\text{sat}}$ versus choline-$Cl_{\text{ext}}$ (-9.3 mV);
- $NMDG_{\text{int}}$ versus choline-$Cl_{\text{ext}}$ (-8.5 mV);
- $TEA_{\text{int}}$ versus choline-$Cl_{\text{ext}}$ (-4 mV);
- $Tris_{\text{int}}$ versus choline-$Cl_{\text{ext}}$ (-5.6 mV);
- $Cs_{\text{int}}$ versus $Cs_{\text{ext}}$ (-2.4 mV);
- $Cs_{\text{int}}$ versus NMDG-$Cl_{\text{ext}}$ (-13 mV);
- $NMDG_{\text{int}}$ versus $Cs_{\text{ext}}$ (1.1 mV);
- $NMDG_{\text{int}}$ versus choline-$Cl(Mg^{2+})_{\text{ext}}$ (-8.8 mV); and
- $Cs_{\text{int}}$ versus choline-$Cl(Mg^{2+})_{\text{ext}}$ (-9.5 mV).

**Statistics**

Data were analyzed with the softwares CLAMPFIT 10.2 (Axon Instruments) and SIGMA PLOT 12 (Systat Software, Chicago, IL). For statistical analysis GRAPHPAD PRISM 5.1 software (GraphPad Software, La Jolla, CA) was used, performing either one-way ANOVA with Bonferroni post-hoc test or Student’s t-test as given. Data are presented as mean $\pm$ SE. Significance level was set to $\alpha$-error lower than $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

**RESULTS**

Ca\textsubscript{v}1.3 voltage sensors are highly sensitive to membrane potential changes but are weakly coupled to activation of ion conductance

To compare the voltage-dependence of charge movement ($Q_{\text{ON}}$-V) with the $I_{\text{Ca}}$-V of different VGCCs we had to establish experimental conditions for reproducible recordings of $Q_{\text{ON}}$ over a large voltage range. This was achieved by using rat Ca\textsubscript{v}1.3 $\alpha_1$-subunits that express at higher levels than human channels (but with indistinguishable current properties (25)) and by replacing Cs$^+$ in our intracellular standard solution with NMDG to prevent contaminating outward currents. Inward currents were blocked by replacing Ca\textsuperscript{2+} with isomolar concentrations of Mg\textsuperscript{2+} and addition of 0.5 mM CdCl\textsubscript{2} and 0.2 mM LaCl\textsubscript{3}. Using a prepulse protocol (Methods; see also below), we have previously shown that the voltage-dependence of $Q_{\text{ON}}$ is not affected by this solution exchange (3) and direct comparisons of $I_{\text{Ca}}$-V and $Q_{\text{ON}}$-V are possible without corrections for surface charge shifts, as discussed in previous studies (24,26). Representative recordings of $I_{\text{Ca}}$ (left panel) and $Q_{\text{ON}}$ (right panel) for Ca\textsubscript{v}1.3\textsubscript{L}, Ca\textsubscript{v}1.2, and Ca\textsubscript{v}3.1 at different test potentials are illustrated in Fig. 1.

As in previous studies with Cs$^+$-solution (3,18,25), the long splice variant of Ca\textsubscript{v}1.3 (Ca\textsubscript{v}1.3\textsubscript{L}) became activated at a voltage range that is more negative than Ca\textsubscript{v}1.2 under identical experimental conditions with NMDG as the major intracellular cation (Fig. 2 A). Ca\textsubscript{v}1.3\textsubscript{L} activated with a half-maximal activation voltage ($V_{0.5}(I_{\text{Ca}})$) of $\sim$10 mV more negative than Ca\textsubscript{v}1.2 (Fig. 2 A; for statistics, see Table 1). To test whether this was due to a more refined voltage sensing, we also measured $Q_{\text{ON}}$-V. Although the threshold voltage for induction of $Q_{\text{ON}}$ was similar for both channels, Ca\textsubscript{v}1.3\textsubscript{L} displayed a steeper voltage-dependence as evident from the significantly lower slope factor and more-negative half-maximal activation voltage of $Q_{\text{ON}}$ ($V_{0.5}(Q_{\text{ON}})$) (Fig. 2 B; and see Table 1 for statistics). A comparison of conductance (G-V) and $Q_{\text{ON}}$-V is shown in Fig. 1 C.

**FIGURE 1** Representative current traces of $I_{\text{Ca}}$ and $Q_{\text{ON}}$. Representative current traces of $I_{\text{Ca}}$ (left) and $Q_{\text{ON}}$ (right) are shown for Ca\textsubscript{v}1.2, Ca\textsubscript{v}1.3\textsubscript{L}, and Ca\textsubscript{v}3.1 at different depolarizing voltages.

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As a measure for the efficiency of voltage-sensor charge movement to pore opening, we plotted the fraction of total observed $Q_{\text{ON}} (Q_{\text{ON}}/Q_{\text{ON,max}})$ required for the activation of $G (G/G_{\text{max}})$ at each voltage (Fig. 2D). Fitting the data to a sigmoid function revealed that significantly less total $Q_{\text{ON}} (52\%, n = 10)$ was required to activate half-maximal conductance of $\text{Ca}_{1.2}$ than of $\text{Ca}_{1.3L} (68\%, n \geq 13)$ (see legend to Fig. 2D). These data indicate weaker coupling of voltage-sensor movements to pore opening in $\text{Ca}_{1.3}$ as compared to $\text{Ca}_{1.2}$. However, the more-sensitive $Q_{\text{ON}}$ charge movement still results in activation at voltages that are more negative than $\text{Ca}_{1.2}$. Low-VGCCs (T-type; $\text{Ca}_{3}$ family (27)) are known to activate at significantly lower voltages than $\text{Ca}_{1.3L} (28,29)$, also shown in our direct comparison with $\text{Ca}_{1.3L}$ (Fig. 2A). This can be explained by the finding that only a small fraction of total $\text{Ca}_{3.1} Q_{\text{ON}} (22\%, n \geq 9; \text{Fig. 2}, B–D)$; and see Table 1) was required for half-maximal activation of conductance. This allows $\text{Ca}_{3.1} I_{\text{Ca}}$ to activate at much lower voltages than $\text{Cav1.3}$ despite its more positive $Q_{\text{ON}}$-V, which is similar to $\text{Ca}_{1.2}$ (Fig. 2B).

Our data show that the voltage-sensing machinery of $\text{Ca}_{1.3}$ responds more sensitively to depolarizing stimuli than $\text{Ca}_{1.2}$ and even $\text{Ca}_{3.1}$. Despite its weaker coupling to pore opening, this allows $\text{Cav1.3} I_{\text{Ca}}$ to carry $I_{\text{Ca}}$ at voltages that are more negative than for $\text{Ca}_{1.2}$.

Alternative splicing affects $Q_{\text{ON}}$ coupling to pore opening of $\text{Ca}_{1.3}$ LTCCs

We next investigated whether the more-negative $I_{\text{Ca}}$-V previously observed for naturally occurring C-terminally short splice variants (such as $\text{Ca}_{1.3}42A$) as compared to the long $\text{Ca}_{1.3L}$ splice variants (18,19) is attributable to more refined voltage sensing or more efficient pore coupling. A negative shift was also observed when intracellular NMDG was used instead of $\text{Cs}^+$ (Fig. 3, A and C) as in previous studies (18,19). The $V_{0.5}(I_{\text{Ca}})$ for $\text{Ca}_{1.3}42A$ was $7 \pm 2$ mV more negative (Fig. 3A; for statistics, see Table 1) and inactivation of $I_{\text{Ca}}$ was faster (due to more pronounced CDI as demonstrated in previous work (18)). Measuring $Q_{\text{ON}}$ for this short splice variant was more
TABLE 1 Biophysical properties of VGCCs α1-subunits

| Cation composition | Voltage (mV) | Slope (mV) | n  |
|--------------------|-------------|------------|----|
| Cav1.2             | -3 ± 2      | 8.6 ± 0.6  | 10 |
| Cav1.3L            | -13 ± 1 *** | 6.4 ± 0.3 ** | 14 |
| Cav1.3L,2A         | -20 ± 2 **   | 5.6 ± 0.3 *** | 17 |
| Cav3.1             | -40 ± 2 ***  | 3.8 ± 0.3 *** | 12 |
| Cav1.2             | 14 ± 2 **    | 11.2 ± 0.5 *** | 28 |
| Cav1.3L            | -4 ± 1      | 8.8 ± 0.2 *** | 19 |
| Cav3.1             | -35 ± 1     | 5.3 ± 0.2 *** | 11 |
| Cav1.2             | -3 ± 2      | 22.6 ± 1.3 | 10 |
| Cav1.3L            | -24 ± 2 *** | 11.6 ± 0.9 *** | 13 |
| Cav3.1             | -6 ± 2 ***  | 19.9 ± 0.9 *** | 10 |
| Cav1.3L            | -29 ± 3     | 10.6 ± 1.9 | 12 |
| Cav1.3L,2A         | -27 ± 2     | 11.1 ± 0.7 | 10 |

Parameters (mean ± SE) were obtained by fitting data of ICa-V relationships or by fitting data of QON-V relationship, as described in methods. QON-V was either determined by measuring QON during pulses to different voltage steps or by measuring QON at Vrev after prepulses to different voltages (V0.5(QON) at Vrev). Statistical significances are indicated for comparison vs. Cav1.2 (*, **, ***), vs. Cav1.3L, (†, ‡, §), and vs. Cav1.3L,2A (†, ‡, §) (one-way ANOVA with Bonferroni post-hoc test). §§, §§§ indicate statistical significance for intra-construct comparisons of parameters obtained with intracellular NMDG vs intracellular Cs (e.g. Cav1.2 choline-Cl/NMDGint vs Cav1.2 choline-Cl/Csint) (Student’s t-test).

difficult than for Cav1.3L due to small (presumably Mg2+) inward currents contaminating the measurement of QON at voltages at ~Vmax, but not at Vrev. We therefore determined QON-V at Vrev (Fig. 3 B) by measuring QON that remained after applying conditioning prepulses to different voltages (see Methods).

Such prepulses moved part of QON in a voltage-dependent manner and allowed calculation of QON-V from the remaining QON at Vrev after the indicated prepulses (QON, post-pre). This protocol has originally been described by McDonough et al. (24) and was validated by us previously (3) and in this study (see legend to Fig. 3 B). Despite the more-negative V0.5(ICa) for Cav1.3L,2A, no significant difference in the QON-V was observed between long and short Cav1.3 constructs (Fig. 3 B; for statistics, see Table 1). G-V curves for both splice variants are shown in Fig. 3 C in relation to their QON-V (for analysis, see Fig. 2 D). Half-maximal G of Cav1.3L,2A required significantly less QON than the long isoform (68 vs. 79%, n ≥ 10) (Fig. 3 D). Taken together, these findings demonstrate that the presence of the intramolecular protein interaction forming the CTM in Cav1.3 LTCCs modulates the ICa-V activation of Cav1.3 by reducing the coupling efficiency between voltage sensing and pore opening.

Cation composition strongly affects voltage-dependent VGCC gating

Comparison of our ICa-V relationships measured using intracellular NMDG (Figs. 2 and 3) with our previously published data employing intracellular Cs+ revealed an ~10 mV shift of V0.5(ICa) toward voltages that were more negative for Cav1.3L (Fig. 4 A). In these experiments, an identical extracellular solution with choline as the major extracellular cation, and 15 mM Ca2+ as the charge carrier, was employed (3,9,18,19,25) (Fig. 4 A). We corrected all data for junction potentials precisely calculated for all our solutions as described in the Methods, ruling out differences in junction potentials as an explanation for this difference. To further characterize this unexpected finding, we also recorded Cav1.2 and Cav1.3L ICa-V relationships with NMDG (NMDGint) or Cs+ containing intracellular solution (Csint). This revealed a negative shift also for these channel types (Table 1) and thus ruled out a Cav1.3-specific effect of NMDG. Next, we tested whether the effect was mimicked by other large organic cations. Tris and TEA caused a similar negative shift of ICa-V like NMDG as shown for Cav1.3L (Fig. 4 D). We also quantified effects on QON for Cav1.3L. Intracellular NMDG also shifted QON-V by approximately the same extent as ICa-V (Fig. 4 B, inset; Cav1.3L; n = 12; Cav1.3L,2A; n = 9; p = 0.02, Student’s t-test), suggesting that the NMDG effect is due to voltage-sensing that is more refined rather than by pore-coupling (Fig. 4 C).

To test whether changes in the voltage-dependence of gating are also observed by corresponding changes of the extracellular solution, we exchanged choline-Cl (in our standard extracellular solution) by either CsCl (Csext) or NMDG-Cl (NMDGext), closely resembling the changes in the internal solutions. The V0.5(ICa) values obtained with the various combinations of intra- and extracellular solutions are illustrated in Fig. 4 D. When intracellular Cs+ was present, the replacement of extracellular choline by NMDG or Cs+ did not cause a change in V0.5(ICa). Intracellular NMDG also caused a negative shift with extracellular Cs+, as observed with extracellular choline. Taken together, our data revealed that intracellular but not extracellular organic cations can enhance the coupling efficiency of Ca2+ channel voltage sensors to membrane depolarization.

DISCUSSION

Our study was motivated by two previous observations regarding Cav1.3 function:

1. Cav1.3L channels were previously reported to activate at lower voltages than Cav1.2. This special feature has been discovered in heterologous expression studies (9,10) but was subsequently confirmed for native Cav1.3 currents in sinoatrial node cells (6), cochlear
inner hair cells (2), and adrenal chromaffin cells (7). It allows Ca_{1.3} channels to sustain subthreshold inward currents and thus serve as a pacemaker channel in the sinoatrial node and chromaffin cells and shape firing patterns of neurons (12).

2. C-terminal splicing removes a CTM (11,18,19), which can further shift the channel’s $I_{Ca} - V$ to more-negative voltages in short splice variants. Because these splice variants are expressed in a tissue-dependent manner, it is likely that they contribute to the fine-tuning of Ca_{1.3} channel activity in different tissues. Here we present data showing that the voltage sensors of Ca_{1.3} respond more readily to depolarization stimuli than those of Ca_{1.2} and Ca_{3.1}. This ensures that despite the weaker coupling of voltage sensing to pore opening, Ca_{1.3} currents can activate at lower membrane potentials than Ca_{1.2}. Moreover, alternative splicing enhances the efficiency of coupling between charge movement and pore opening, explaining the even lower activation voltage range of naturally occurring short splice variants lacking the CTM.

Based on contemporary structural models of the voltage-gated cation channel family, mainly derived from x-ray structures of voltage-gated K\textsuperscript{+}-(30) and bacterial Na\textsuperscript{+}-channels (31), a negative shift in the voltage-dependence of channel conductance ($G - V$) may, in principle, result via two possible mechanisms:

1. Values of $Q_{ON} - V$ that are more negative. Even if the efficiency of voltage-sensor coupling to the pore remains unchanged, this should shift $G - V$ to voltages that are more negative. An example is the deletion of a ‘gating brake’ in T-type channel α_{1c}-subunits. This causes a negative shift and steeper voltage-dependence (slope) of the $Q_{ON} - V$ relationship (29) paralleled by a corresponding negative shift in $G - V$ (32).

2. $Q_{ON} - V$ is unaltered but the efficiency of coupling to pore opening is enhanced. This has been reported for the LTCC activators FPL64176 and BayK8644, which induce strong

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**FIGURE 3**

$I_{Ca}$ and $Q_{ON}$ voltage-dependence of Ca_{1.3}L in comparison to Ca_{1.3}42A. The n-numbers and detailed statistics are given in Table 1. (A) $I_{Ca} - V$ of Ca_{1.3}L and Ca_{1.3}42A recorded with standard recording solutions (choline-Cl/NMDG-int). Fits were generated as described in Fig. 2 A. (B) $Q_{ON} - V$ measured for Ca_{1.3}L (n = 12) and Ca_{1.3}42A (n = 10) using the prepulse protocol (inset) as described in Methods with Mg\textsuperscript{2+}-containing solution. (Inset) Example traces for both constructs obtained by depolarization to the reversal potential after a prepulse to −28.8 mV, which causes partial movement of $Q_{ON}$. The value $Q_{ON}$ after the prepulse ($Q_{ON, post-pre}$) was normalized to $Q_{ON, max}$. For Ca_{1.3}L the same gating parameters were obtained using this and the protocol in Fig. 2 B. (C) Comparison of the $Q_{ON} - V$ and $G - V$ relationships of Ca_{1.3}L and Ca_{1.3}42A (Solid and dashed lines) Boltzmann fits of $Q_{ON} - V$ data obtained from experiments illustrated in panel B (dotted line is the same as in Fig. 2 C (Ca_{1.3}L) for comparison). (D) Data representation as in Fig. 2 D. Data points were obtained from the experiments given in Table 1. Pooled data were fitted to a sigmoid function to determine the fractional $Q_{ON}$ at half-maximal $G$. Values were significantly different between the two splice variants (determined by sum-of-squares F-test using GRAPHPAD PRISM, GraphPad Software).
changes in the kinetics and gating of Cav1.2 currents (24,33), including a shift of the $I_{\text{Ca}}-V$ relationship to potentials that are more hyperpolarized (33). However, they do not affect the voltage-dependence of $Q_{\text{ON}}$ (24,33).

Here we clearly demonstrate that the more-negative activation range of Cav1.3 as compared to Cav1.2 is not due to a more efficient coupling. Instead, coupling is even weaker, as evident from a higher fractional $Q_{\text{ON}}$ required for $V_{0.5}(I_{\text{Ca}})$. However, $Q_{\text{ON}}$ of Cav1.3 displayed a steeper voltage-dependence and thereby still permits a more-negative $I_{\text{Ca}}-V$. We show that this is in contrast to the mechanism imposed by alternative splicing. We found that in the absence of the CTM, the $Q_{\text{ON}}-V$ does not change but more channel activation (i.e., fractional conductance) is observed at a given percentage of maximal $Q_{\text{ON}}$. Similar to the findings obtained with FPL64176 (24), this can be interpreted as more efficient coupling between voltage-sensor movements and pore opening.

It is unclear how the CTM can moderate this coupling. Molecular studies using mutant and chimeric channel constructs will be difficult to perform because this modulatory domain is part of a larger structure consisting not only of the two noncovalently interacting putative $\alpha$-helices (PCRD, DCRD) of the modulatory domain itself but also of channel-bound CaM (18,34). Based on our observations, it is most likely that the CTM targets the interaction of pore-forming helices (primarily S6) with the S4-S5 linker. Such an interaction, which is also modulated by A-kinase anchoring proteins, has recently been described in Cav1.2 $\alpha_{1}$-subunits (37).

What structural differences mediate the steeper voltage-dependence of Cav1.3 and the higher coupling efficiency of Cav3.1? The amino-acid sequence within the S4 segments including the positive charges of Cav1.3 and Cav1.2 are highly conserved (see alignment in Fig. S1 in the Supporting Material) and are unlikely to explain the difference in their $Q_{\text{ON}}-V$ relationships. Structural features outside S4 must therefore play a crucial role. Mutations in S6 segments forming the activation gate or the S5-S6 linker, which couples voltage-sensors to the gate, can induce $Q_{\text{ON}}-V$ shifts.

FIGURE 4 Effect of intracellular cations on Cav1.3L voltage-dependence. (A) $I_{\text{Ca}}-V$ of Cav1.3L recorded with either $C_{\text{int}}$ or NMDG$_{\text{int}}$. (For comparison, $I_{\text{Ca}}-V$ of Cav1.3L recorded with NMDG$_{\text{int}}$ is shown as a line, as taken from Fig. 2A.) Fits were generated as described in Fig. 2A. For statistics, see panel D. (B) $Q_{\text{ON}}-V$ of Cav1.3L recorded at $V_{\text{rev}}$ as in Fig. 3 B with either Cs$^+$ ($C_{\text{int}}$) or NMDG (NMDG$_{\text{int}}$) as the major intracellular cation. For comparison, the $Q_{\text{ON}}-V$ of Cav1.3L in NMDG$_{\text{int}}$ is illustrated (line taken from Fig. 3 B). (Inset) Statistical comparison of the $V_{0.5}$ values ($C_{\text{int}}$: $-20 \pm 1$ mV, $n = 9$; NMDG$_{\text{int}}$: $-29 \pm 3$ mV, $n = 12$; $p = 0.022$, Student’s t-test). (C) Normalized $G/G_{\text{max}}$ to normalized $Q_{\text{ON}}/Q_{\text{ON}, \text{max}}$ (replotted from Fig. 3 D as 1-$Q_{\text{ON}}/Q_{\text{ON}, \text{max}}$). (D) $V_{0.5}(I_{\text{Ca}})$ values for Cav1.3L, recorded with different internal and external cation-based solutions, as indicated (for solution composition, see Methods) ($n \geq 9$). For calculation of statistical significance, one-way ANOVA with Bonferroni post-hoc test was performed ($p < 0.05$, *; $p < 0.005$, **; $p < 0.001$, ***).
to voltages that are more negative. This has been reported by us (for Ca\textsubscript{a,1.3} (3)) and others (for Ca\textsubscript{a,3.2} and Ca\textsubscript{a,2.3} (35,38)). Interdomain cytoplasmic linkers (shown for N-terminal regions of the I-II loop in the case of Ca\textsubscript{a,3} channels) can also affect the voltage-dependence of \(Q_{ON}V\) by serving as a \textquoteleft gating brake\textquoteright\ (29). The high sequence similarity of Ca\textsubscript{a,1.3} a\textsubscript{1} subunits with Ca\textsubscript{a,1.2} also outside S4 regions provides an excellent opportunity to identify the structural determinants accounting for its uniquely steep voltage-dependence using chimeric approaches.

The high coupling efficiency of Ca\textsubscript{a,3.1} channels is also not readily explained by charge differences in the S4 segments (see Fig. S1). Assuming that all four voltage sensors have to move completely for pore opening, the fact that only ~25% of \(Q_{ON}\) charge are moved when conductance is already fully activated (Fig. 2) could indicate that the activation of only one of the four voltage sensors is sufficient for activating the channel gate.

During the course of our studies, we also discovered that intracellular organic cations sensitize voltage-responses of all three VGCCs investigated. A shift to more negative \(I_{Ca}\) was initially observed when intracellular Cs\textsuperscript{+} was replaced by NMDG, but was also found for Tris and TEA. In contrast to splicing, NMDG affected \(Q_{ON}V\) with no major change in pore coupling, suggesting that it primarily affects voltage sensing itself. This also demonstrates that ion permeation is not required for this voltage shift. This modulation is unlikely to have been caused by a high affinity interaction with NMDG; it was not observed when only 15 mM of Cs\textsuperscript{+} were replaced by NMDG (\(n = 4\), not shown).

To investigate the possibility of passive charge screening effects, we measured changes in Ca\textsubscript{a,1.3}\textsubscript{L} gating with all possible combinations of equimolar concentrations of NMDG and Cs\textsuperscript{+} in the intra- and extracellular solutions. Considerably less is known about passive-charge-screening effects of organic cations as compared to mono- or divalent inorganic cations (39–43). Therefore, although unlikely, the possibility of passive charge screening effects cannot be completely excluded. Alternatively, intracellular organic cations may somehow more specifically interfere with the gating apparatus. An example has previously been described for K\textsubscript{a,1.2} channels (44) by showing that internal cations are able to occupy the inner cavity of the open channel. Thereby they prevent closing of the inner pore gate and stabilize the open state of the voltage sensors (44). This was not associated with a shift in \(Q_{ON}V\), but caused a slowing of off-gating currents, especially with such larger cations as NMDG and TEA. However, we were not able to detect a major off-gating current-stabilizing effect of NMDG on Ca\textsubscript{a,1.3}\textsubscript{L} (\(n \geq 9\), not shown) or a slowing of \(I_{Ca}\) deactivation kinetics.

Independent from the molecular mechanism, our findings clearly emphasize that recording buffer compositions have to be considered when comparing biophysical parameters across different studies examining the voltage-dependent gating properties of VGCCs.

**SUPPORTING MATERIAL**

One figure is available at http://www.biophysj.org/biophys/supplemental/S0006-3495(14)00222-7.

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