Modulation of Voltage- and Ca\(^{2+}\)-dependent Gating of Ca\(_V\) 1.3 L-type Calcium Channels by Alternative Splicing of a C-terminal Regulatory Domain*

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Low voltage activation of Ca\(_V\) 1.3 L-type Ca\(^{2+}\) channels controls excitability in sensory cells and central neurons as well as sinoatrial node pacemaking. Ca\(_V\) 1.3-mediated pacemaking determines neuronal vulnerability of dopaminergic striatal neurons affected in Parkinson disease. We have previously found that in Ca\(_V\) 1.4 L-type Ca\(^{2+}\) channels, activation, voltage, and calcium-dependent inactivation are controlled by an intrinsic distal C-terminal modulator. Because alternative splicing in the Ca\(_V\) 1.3 α1 subunit C terminus gives rise to a long (Ca\(_V\) 1.3\(_{42}\)) and a short form (Ca\(_V\) 1.3\(_{42A}\)) channels, we investigated if a C-terminal modulatory mechanism also controls Ca\(_V\) 1.3 gating. The biophysical properties of both splice variants were compared after heterologous expression together with a C-terminal modulator in short splice forms facilitates Ca\(_V\) 1.3 channel activity by alternative splicing. The absence of the C-terminal modulatory mechanism also controls Ca\(_V\) 1.3 C terminus of Ca\(_V\) 1.3\(_{42}\) and a short form (Ca\(_V\) 1.3\(_{42A}\)) was investigated if a C-terminal modulatory mechanism also controls Ca\(_V\) 1.3 gating. By investigating several Ca\(_V\) 1.3 channel truncations, we restricted the modulator activity to the last 116 amino acids of the C terminus. The resulting Ca\(_V\) 1.3\(_{ΔC116}\) channels showed gating properties similar to Ca\(_V\) 1.3\(_{42A}\) that were reverted by co-expression of the corresponding C-terminal peptide C\(_{116}\). Fluorescence resonance energy transfer experiments confirmed an intramolecular protein interaction in the C terminus of Ca\(_V\) 1.3 channels that also modulates calmodulin binding. These experiments revealed a novel mechanism of channel modulation enabling cells to tightly control Ca\(_V\) 1.3 channel activity by alternative splicing. The absence of the C-terminal modulator in short splice forms facilitates Ca\(_V\) 1.3 channel activation at lower voltages expected to favor Ca\(_V\) 1.3 activity at threshold voltages as required for modulation of neuronal firing behavior and sinoatrial node pacemaking.

Ca\(^{2+}\) influx through voltage-gated L-type Ca\(^{2+}\) channels (LTCCs) regulates numerous physiological functions, including muscle contraction, hormone release, neuronal firing and plasticity, sensory function, and cardiac pacemaking (1). Four pore-forming α1 subunit isoforms (Ca\(_V\) 1.1–Ca\(_V\) 1.4) with different biophysical properties and expression profiles evolved to adjust LTCC Ca\(^{2+}\) signals to cellular needs. Ca\(_V\) 1.3 channels play a unique role for hearing (sensory signaling in cochlear inner hair cells (IHC)) and sinoatrial node (SAN, diastolic depolarization) function (2). Ca\(_V\) 1.3 channels appear ideally suited for these functions because the operating range of both IHCs and SAN cells is within a voltage range of about −60 and −40 mV, where these channels can conduct inward current because of their negative activation range (Refs. 3–5 and for review see Ref. 6). In neurons, Ca\(_V\) 1.3 channels also serve pacemaker function and shape neuronal firing (7, 8). Ca\(^{2+}\) influx through LTCCs is limited by a Ca\(^{2+}\)-dependent feedback mechanism, the so-called calcium-dependent inactivation (CDI) (9). CDI develops in response to local or global elevations of intracellular Ca\(^{2+}\) sensed by channel-bound calmodulin (10, 11). However, moderation of CDI is an important prerequisite in some cells, such as in IHCs (Ca\(_V\) 1.3; 12–14) and in retinal photoreceptors (Ca\(_V\) 1.4), where slow inactivation of \(I_{\text{Ca}}\) is required for sensory signaling. In Ca\(_V\) 1.4 channels, CDI is completely prevented by an intrinsic gating modulator located in the Ca\(_V\) 1.4\(_{14}\) C terminus, and CDI is restored in C-terminal truncation mutants (15, 16). This C-terminal gating modulator (CTM) not only prevents CDI but also shifts the activation voltage range to more positive potentials (15). Gating modulation seems not to be limited to Ca\(_V\) 1.4 because co-expression of C-terminal fragments of Ca\(_V\) 1.2 channels also shifts their activation range to more positive voltages and inhibits pore opening (17). Because C termini of Ca\(_V\) 1.2 (and also Ca\(_V\) 1.1) α1 subunits are proteolytically cleaved (18–20) but remain noncovalently bound to

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2 The abbreviations used are: LTCC, L-type Ca\(^{2+}\) channel; ANOVA, analysis of variance; GFP, green fluorescent protein; YFP, yellow fluorescent protein; EFYP, enhanced YFP; CFP, cyan fluorescent protein; RT, reverse transcription; FRET, fluorescence resonance energy transfer; CTM, C-terminal gating modulator; CDI, calcium-dependent inactivation; DCRD, distal C-terminal regulatory domain; VDF, voltage-dependent facilitation; fwd, forward; rev, reverse; IHC, inner hair cells; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; SAN, sinoatrial node; CaM, calmodulin; PCRD, primal C-terminal regulatory domain.
proximal C-terminal domains, they may serve as autoinhibitory modules (17, 21).

We hypothesized that CaV1.3 function may also be fine-
tuned by a CTM domain similar to CaV1.1, CaV1.2, and CaV1.4. Unlike for other LTCC &alpha; subunits, alternative splicing gener-
ates CaV1.3 &alpha; subunits with long (usage of exon 42, CaV1.3 42a) or short (usage of exon 42A, CaV1.3 42A) C termini. A CTM would therefore confer different biophysical properties on CaV1.3 only if present as in certain (i.e. long) variants.

Here we provide unequivocal evidence that an intramole-
cular protein-protein interaction gives rise to substantial gating differences between CaV1.3 42 and CaV1.3 42A. Our finding that the long and the short splice variants are expressed in several mouse and human tissues suggests that both contribute to fine-
tuning of physiological CaV1.3 channel function.

**EXPERIMENTAL PROCEDURES**

**Cloning of cDNA Constructs**

To generate splice variant CaV1.3 42A, the alternatively spliced exon 42A was introduced into CaV1.3 cDNA (CaV1.3 42 = human CaV1.3 42A as in Ref. 4, GenBank accession number EU363339) by co-ligating an EcoRI-BamHI-cut PCR product containing exon 42A from a rat brain &alpha; subunit (GenBank accession number M57682) with HindIII-EcoRI-cut CaV1.3 cDNA into HindIII-BamHI (4622–7100)-cut CaV1.3-pBS vector. The rat exon 42A amino acid sequence is identical to its human homologue. Human CaV1.3 containing exon 42A was subsequently cloned into vector pGFP (wild type) short form of the CaV1.3 protein (4). For analyzing the effects of exon 44, amino acid residues RTRYETY1 were introduced into an untagged construct containing the long CaV1.3 C terminus after position 1787 using standard PCR techniques. CaV1.3 3αc477p, CaV1.3 3αc158p, CaV1.3 3αc116p and CaV1.3 3αc76p channels were constructed by introducing stop codons into untagged CaV1.3 3αs at respective amino acid positions 1665, 1980, 2022, and 2062 followed by artificial restriction sites as follows: BamHI for CaV1.3 3αc158 and CaV1.3 3αc76, HpaI* for CaV1.3 3αc116 and XhoI* for CaV1.3 3αc473. Peptides GFP-C158p, GFP-C116p, and GFP-C76p were constructed by ligating HindIII-Sall*-flanked PCR products corresponding to amino acids 1980–2137, 2022–2137, and 2062–2137 of CaV1.3 into the pGFP plasmid resulting in the corresponding GFP-tagged CaV1.3 C-terminal peptides (22). For FRET analysis, all C-termi-

nal CaV1.3 fragments were cloned either into the mammalian expression vector pEYFP-C1 for N-terminal EYFP labeling or vector pECFP-N1 for C-terminal enhanced GFP labeling (Clontech). N-terminally EYFP-labeled constructs 5'-HindIII* and 3'-SalI* restriction sites flanking nucleotide sequences corresponding to the following CaV1.3 amino acid positions were introduced and cloned into HindIII-Sall-cut vector pEYFP-C1 as follows: peptide EF-preIQ-IQ-postIQ, amino acids 1453–2137; EF-preIQ-IQ, amino acids 1453–1623; and EF-preIQ-IQ-PCRDR, amino acids 1453–1664. To construct peptide EF-preIQ-IQ-PCRDRREEQQ, arginines at positions 1640 and 1641 were mutated to glutamines by splicing by overlapping exten-

**Qualitative RT-PCR**

Mouse tissue was prepared from C57B/6N wild-type mice. Total RNA was isolated from whole-brain and brain sub-re-

regions using the RNAqueos®-4PCR kit (Ambion, Foster City, CA). After DNase I treatment, RNA integrity was checked by the quality of the 28 S and 18 S rRNA bands on a denaturing agarose gel. Organ of Corti mRNA was prepared from P4 or P19 mice as described in Ref. 23. Human total RNAs from brain, heart, pancreas, and retina were purchased from Clontech. One μg of total RNA was used for first strand cDNA synthesis by reverse transcriptase (RevertAid™ H M Inus First Strand cDNA synthesis kit, MBI; Fermentas, Hanover, Germany) and random hexamer primers. For PCR analysis, the forward (fwd) primer was located in exon 39 (GenBank accession number NM_028981 for mouse and EU363339 for human). To differ-

entiate between CaV1.3 42 and CaV1.3 42A, specific reverse (rev) primers were designed, located either in exon 42 or 42A. The following primers were used for mouse: fwd primer, 5’-CAACCCCTTGGTTTTGCTTC-3’ and rev primer for exon 42, 5’-TATAGCCCGCTGGATTCTGG-3’ (332-bp product); rev primer for exon 42A, 5’-CTTCTCCCGGAGGAGTGC-3’ (483-bp product). The following primers were used for human: 5’-AACCTGTGTGTGTGGTTC-3’ (387-bp product) and rev primer for exon 42, 5’-TATAGCCCGCTGGATTCTGG-3’ (331-bp product); rev primer for exon 42A, 5’-CCACCTTCC-

GGAGGATG-3’ (488-bp product). In mouse organ of Corti, splice variants were also detected with the following primer pairs: exon 42 fwd primer, 5’-CTGCGGTACCGATGTGGTTCCCTCCA-3’ and rev primer, 5’-CTACAGGTGGTAATGC

AAATCAT-3’; exon 42A, fwd primer, 5’-CGCCGATCCGTACATTGGGACAGTTGTTCC-3’; rev primer, 5’-CGCAAGGC

TTCTAGGACATCTGGTCAAGC-3’. Mouse and human GAPDH was used as positive control as follows: for mouse (GenBank accession number NM_008084), fwd primer, 5’-ACTCCACTCACCGCAAAATTC-3’ and rev primer 5’-CACATTTGGGGTGAAGGAAC-3’ (572-bp product); for human (GenBank accession number NM_002046), fwd primer, 5’-CAATGACCCCTTCATTGACC, rev primer, 5’-CTACAGGTGGTAATGC

AAATCAT-3’; exon 42A, fwd primer, 5’-CGCCGATCCGTACATTGGGACAGTTGTTCC-3’; rev primer, 5’-CGCAAGGC

TTCTAGGACATCTGGTCAAGC-3’. Mouse and human GAPDH was used as positive control as follows: for mouse (GenBank accession number NM_008084), fwd primer, 5’-ACTCCACTCACCGCAAAATTC-3’ and rev primer 5’-CACATTTGGGGTGAAGGAAC-3’ (572-bp product); for human (GenBank accession number NM_002046), fwd primer, 5’-CAATGACCCCTTCATTGACC, rev primer, 5’-CGCCGATCCGTACATTGGGACAGTTGTTCC-3’; rev primer, 5’-CGCAAGGC

TTCTAGGACATCTGGTCAAGC-3’. Mouse and human GAPDH was used as positive control as follows: for mouse (GenBank accession number NM_008084), fwd primer, 5’-ACTCCACTCACCGCAAAATTC-3’ and rev primer 5’-CACATTTGGGGTGAAGGAAC-3’ (572-bp product); for human (GenBank accession number NM_002046), fwd primer, 5’-CAATGACCCCTTCATTGACC, rev primer, 5’-CGCCGATCCGTACATTGGGACAGTTGTTCC-3’; rev primer, 5’-CGCAAGGC

TTCTAGGACATCTGGTCAAGC-3’.
of 1.5 mM MgCl₂. The resulting PCR products were subcloned into pGEM-T Easy vectors (Promega, Mannheim, Germany) and sequenced.

**Quantitative RT-PCR**

RNA isolation and cDNA preparation—Tissue from three adult male C57BL/6N mice (3–6 months) was used for whole-brain and brain sub-region cDNA preparations. Total RNA and cDNA were prepared as described (24).

Quantitative RT-PCR—The relative abundance of different Ca₃.1 mrnas was assessed by TaqMan quantitative PCR (50 cycles) using a standard curve method as described (24). Taq-Man gene expression assays, designed to span exon-exon boundaries, were purchased from Applied Biosystems (Foster City, CA). Assessment of mouse GAPDH transcripts was included as efficiency reference. Assay identification numbers and sequences were provided as efficiency reference. Assay identification numbers were prospectively excluded from analysis of activation and inactivation parameters to guarantee high quality voltage clamp. CDI was quantified as the current remaining at the end of 250-ms depolarizations to different test potentials, expressed as fraction of peak current amplitude (I/250). Parameter f was defined as the difference between I/250 values of Iₚ and Iₐ at +10 mV. CDI experiments were performed on different cells, but effects were the same when the charge carrier was switched from Ba²⁺ to Ca²⁺ on the same cell in representative experiments. All voltages were corrected for the liquid junction potential.

**Confocal FRET Microscopy**

Confocal FRET microscopy was performed as described previously (15). As negative controls, peptide YFP-EF-PreIQ-PCRD was co-expressed with CFP alone (CFP control), and probes CFP-C135 or CFP-C116 with YFP alone (YFP controls 1 and 2). Image analysis was accomplished using a self-made program integrated in MatLab 7 based on the FRET analysis algorithm proposed (26). Interaction between fragments was considered when a significant difference to the CFP as well as the corresponding YFP control was observed. The local ratio between CFP and YFP might vary because of different local expression levels of the different protein constructs, which could affect calculation of FRET values. Therefore, analysis was limited to pixels with a CFP:YFP molar ratio between 1:10 and 10:1.

**Statistical Analysis**

Data were analyzed using Clampfit 9.0 (Molecular Devices, Union City, CA) and Microcal Origin 5.0 (Northampton, MA). Significant difference between the two splice variants was assessed a priori by pairwise comparison before other truncation constructs to characterize the modulatory mechanism were made. Student’s t test or Mann-Whitney test were used for comparison between two groups for parametric and nonparametric data, respectively. Statistical significance was determined using one-way ANOVA followed by Bonferroni post-test, except if stated otherwise. Statistical significance was set at p < 0.05. All data are presented as mean ± S.E. for the indicated number of experiments.

**RESULTS**

Ca₃.1.3 C-terminal Modulator Affects Voltage-dependent Channel Activity—To test for the presence of a C-terminal modulator (CTM), we employed two previously described nat-
CaV1.3 C-terminal Gating Modulation

![Diagram](image)

**FIGURE 1.** C-terminal splice variants CaV1.342 and CaV1.342A. a, alternative usage of exon 42 results in either full-length (exon 42, black) or C-terminally truncated (exon 42A, gray) CaV1.3 channels. Arrows indicate the approximate position of forward (fwd) and reverse (rev) PCR primers used in Fig. 8. b, immunoblot of both splice variants expressed together with β3 and α2δ1 subunits in HEK-293 cells, as described under “Experimental Procedures.” Mock-transfected cells (mock) were used to show specificity of immunoreactivity. One representative experiment out of four is shown.

![Diagram](image)

**FIGURE 2.** Activation and steady-state inactivation properties of CaV1.342 compared with CaV1.342A channels. a, normalized mean I-V curves of I_{Ca} for CaV1.342 (black) and CaV1.342A (gray) channels. Activation parameters and statistics are given in Table 1. The dotted line describes the fit to a normalized I-V curve of I_{Ca} for CaV1.2 in comparison: V_{0.5,act} = 8.8 mV; k_{act} = 11.0 mV. Cell, 111d. b, representative current traces for I_{Ca} (300-ms depolarization to indicated test potentials). Cells: CaV1.342: 196I_23; CaV1.342A: 196I_32. c, V_{0.5,act} for CaV1.342A was independent from current density. d, exemplar ON-gating currents for CaV1.342 and CaV1.342A with similar I_{ba} amplitudes measured by depolarizing cells to V_{m}. One representative out of six experiments is shown. Cells: CaV1.342: 207l_34; CaV1.342A: 237k_26. e, steady-state inactivation curves for CaV1.342 and CaV1.342A. Activation curves were obtained from parameters in a. Solid lines are fits to the Boltzmann relationship (see “Experimental Procedures”). Statistics are given under “Results.” Error bars reflect 5E.

Uirally occurring CaV1.3 α1 subunit splice variants, CaV1.3_{42} and CaV1.3_{42A} (also termed CaV1.3a and CaV1.3b, respectively (5, 27, 28)), which differ with respect to the length of their C termini, as illustrated in Fig. 1a. Similar to the CaV1.4 transduction mutant K1591X (15), CaV1.3_{42A} terminates shortly after the IQ motif and lacks most of a homologous region (termed PCRD) previously found to participate in the binding of distal C-terminal sequences in CaV1.2 (17). Wild-type long and short CaV1.3 α1 subunits were expressed in HEK-293 cells together with β3 (or β2a) and α2δ1. β3 subunits were selected because we have previously shown that they form a large fraction of dihydropyridine-sensitive LTCCs in the brain (29). Both α1 subunits migrated with the expected molecular mass in Western blots of HEK-293 cell preparations (Fig. 1b).

We systematically investigated the biophysical properties of both I_{Ca} and I_{ba} for CaV1.3_{42} and CaV1.3_{42A} as illustrated in Figs. 2 and 3 and Tables 1–3. The voltage dependence of I_{Ca} activation of CaV1.3_{42A} was significantly shifted to hyperpolarizing potentials by about 10 mV (CaV1.3_{42}: V_{0.5,act} = −2.2 ± 0.6 mV, n = 14; CaV1.3_{42A}: V_{0.5,act} = −12.9 ± 0.8 mV, n = 15; p < 0.001; see Table 1). This negative shift was because of the significant decrease in k_{act} (CaV1.3_{42}: −9.1 ± 0.2, n = 14; CaV1.3_{42A}: −6.9 ± 0.2, n = 15; p < 0.001; see Table 1) without change in the activation threshold (Fig. 2a). Such changes were also observed for I_{ba} (Table 2). The negative activation range persisted when subgroups of cells with different current amplitudes (0.1–0.6 versus 0.7–2.3 nA) were compared (Fig. 2c). It was not restricted to β3 subunit-containing channel complexes because it was also seen when the CaV1.3 splice variants were co-expressed with β2a subunits (V_{0.5,act} I_{Ca}: CaV1.3_{42}: 0.06 ± 3.2 mV, n = 3; CaV1.3_{42A}: V_{0.5,act} = −11.2 ± 1.7 mV, n = 3; I_{ba}: CaV1.3_{42}: −12.5 ± 1.6 mV, n = 7; CaV1.3_{42A}: −22.7 ± 1.3 mV, n = 6). Mean current density of CaV1.3_{42A} channels was 2.5-fold higher for I_{Ca} (CaV1.3_{42}: 21.0 ± 3.3 pA/pF, n = 14; CaV1.3_{42A}: 98.3 ± 31.8, n = 19, p < 0.01, Mann-Whitney test). A similar increase was observed for I_{ba} (not shown). Increased current density occurred without detectable changes in the expression density as observed in immunoblots...
Biophysical \( I_{\text{Ca}} \) properties of Ca\(_{\text{v}}\).1.3 and Ca\(_{\text{v}}\).1.3.42A channels in comparison with different C-terminally truncated Ca\(_{\text{v}}\).1.3 channel constructs

Number of experiments is indicated in parentheses.

| Construct | \( V_{\text{0.5,act}} \) | \( k_{\text{act}} \) | \( V_{\text{max}} \) | Activation threshold | \( V_{\text{rev}} \) |
|-----------|--------------------|-----------------|-----------------|---------------------|----------------|
| Ca\(_{\text{v}}\).1.3 (14) | -2.2 ± 0.6 \( ^a \) | -9.1 ± 0.2 \( ^a \) | 13.2 ± 0.4 \( ^a \) | -35.6 ± 0.7 | 69.8 ± 1.1 |
| Ca\(_{\text{v}}\).1.3.42A (15) | -12.9 ± 0.8 \( ^a \) | -6.9 ± 0.2 \( ^a \) | 1.9 ± 0.9 \( ^a \) | -37.1 ± 0.8 | 65.7 ± 1.1 |
| Ca\(_{\text{v}}\).1.3.42A + C\(_{158}\) (7) | -12.2 ± 1.5 \( ^a \) | -7.6 ± 0.3 \( ^a \) | 2.1 ± 1.2 \( ^a \) | -38.9 ± 0.7 | 63.6 ± 2.2 |
| Ca\(_{\text{v}}\).1.3.42A + C\(_{156}\) (7) | -0.8 ± 1.4 \( ^a \) | -9.7 ± 0.4 \( ^a \) | 13.9 ± 1.2 \( ^a \) | -36.5 ± 0.9 | 67.5 ± 1.2 |

\( ^a \) Values are \( p < 0.0001 \) compared with Ca\(_{\text{v}}\).1.3.42A (one-way ANOVA followed by Bonferroni post-test).

\( ^b \) Values are \( p < 0.001 \) (statistically significant from Ca\(_{\text{v}}\).1.3).

\( ^c \) Values are \( p < 0.01 \).

\( ^d \) Values are \( p < 0.05 \).

(1b, \( n = 4 \)). Depolarization to the reversal potential (\( V_{\text{rev}} \)) revealed a small ON-gating current which was always detectable for Ca\(_{\text{v}}\).1.3.42A channels but was absent (or much reduced) in Ca\(_{\text{v}}\).1.3.42A currents (Fig. 2d). This further suggests that the larger current amplitude of Ca\(_{\text{v}}\).1.3.42A channels was not because of an increased expression density of channels at the plasma membrane in line with the immunoblot analysis.

The short C terminus also resulted in a slight decrease of \( V_{\text{rev}} \) (with a larger and statistically significantly different for \( I_{\text{Na}} \); see Tables 1 and 2). The half-maximal voltage of steady-state inactivation (\( V_{\text{0.5,inact}} \)) induced by 10-s conditioning pulses (2e) for the physiological charge carrier was also significantly shifted toward more negative voltages in Ca\(_{\text{v}}\).1.3.42A channels (Fig. 3). The difference was most pronounced during the first 30 ms (% inactivation: Ca\(_{\text{v}}\).1.3.42A, 98.1 ± 0.3%; Ca\(_{\text{v}}\).1.3, 73.6 ± 1.9%; \( n = 19 \); \( p = 0.0006 \); for multiple comparison test, see Table 3). Inactivation included an initial fast component followed by a slow component in both isoforms.

**Ca\(_{\text{v}}\).1.3 C Terminus Modulates Ca\(^{2+}\)-dependent Inactivation Properties**—As already obvious from the representative current traces in Fig. 2b, Ca\(_{\text{v}}\).1.3.42A channels inactivate faster during depolarization to different test potentials than Ca\(_{\text{v}}\).1.3.42A channels. This is also evident from a detailed comparison of \( I_{\text{Ca}} \) inactivation during 10-s pulses to the maximum of the I-V curve (\( V_{\text{max}} \); Table 3). The difference was most pronounced during the first 30 ms (% inactivation: Ca\(_{\text{v}}\).1.3.42A, 98.1 ± 0.3%; Ca\(_{\text{v}}\).1.3, 73.6 ± 1.9%; \( n = 19 \); \( p = 0.0006 \); for multiple comparison test, see Table 3). Inactivation included an initial fast component followed by a slow component in both isoforms.

The window current toward more hyperpolarizing voltages for Ca\(_{\text{v}}\).1.3.42A (Fig. 2e). Our data demonstrate that the structural divergence within the C termini of these Ca\(_{\text{v}}\).1.3 splice variants causes pronounced changes in channel gating suggesting the existence of a CTM in Ca\(_{\text{v}}\).1.3 LTCCs that controls their activity at negative voltages.
The faster inactivation of $I_{Ca}$A could be due to faster voltage-dependent inactivation, faster CDI, or both. To address this question, we also quantified $I_{Ca}$A-mediated Ca and Ba inactivation properties of CaV1.3 and CaV1.3A channel-mediated currents. $I_{Ba}$ remaining after 250 ms; f is the difference between $r_{250}$ values at $+10$ mV (for statistics see “Results” and Table 3). Number of experiments is given in parentheses. Error bars reflect S.E.

The faster inactivation of CaV1.3A could be due to faster voltage-dependent inactivation, faster CDI, or both. To address this question, we also quantified CaV1.3-mediated $I_{Ba}$ of both splice variants. Interestingly, mean $I_{Ba}$ inactivation was even slower for CaV1.3A channels (Fig. 3d and Table 3). This difference was because of a pronounced slowing of inactivation in 50% of the cells (% inactivation of CaV1.3A during 250 ms, 8.6 ± 1.2%, n = 7, in this subset). We have previously observed a similar slowing of inactivation of $I_{Ba}$ in truncated CaV1.4 channels (15).

From these data we conclude that the faster inactivation of $I_{Ca}$A observed for CaV1.3A must be due to more pronounced CDI. As a quantitative measure of CDI, we analyzed the fraction of $I_{Ca}$ through CaV1.3 channels remaining after a 250-ns depolarization ($r_{250}$) in a wide voltage range (Fig. 3e). A typical U-shaped dependence on test voltage was observed for both splice variants, but it was more pronounced for CaV1.3A channels ($f = 0.60 ± 0.07$ versus $0.25 ± 0.11$ at 10 mV for CaV1.3A channels). In agreement with a previous report (13), CaV1.3 CDI was CaM-mediated. $I_{Ca}$A inactivation of CaV1.3A channels was dramatically slowed by co-expression of YFP-labeled dominant-negative CaM (CaM1234) ($r_{250}$ at $V_{max}$: CaV1.3, 16.3 ± 0.5 mV; CaV1.3A: 7.4 ± 0.6 mV, n = 6) versus 16.3 ± 0.5 mV, n = 8) suggesting that the shift in activation voltage occurs independent of CaM as also shown previously for CaV1.4 (15). Taken together, these data provide unequivocal evidence that the CTM in CaV1.3 channels not only modulates its activity range but also moderates CDI. CaV1.3 CTM Lies within the Most Distal C-terminal Domain Highly Conserved in LTCCs—To further strengthen this hypothesis and pinpoint important structural domains, we tested if different C-terminal truncations exhibit similar gating effects. In accordance to the C-terminal peptide C122 important for CaV1.4 channel modulation (15), we deleted the corresponding distal 158 amino acids of CaV1.3A. The resulting CaV1.3C158 channels exhibited $I_{Ca}$ and $I_{Ba}$ activation properties comparable with CaV1.3A (Tables 1 and 2). For $I_{Ca}$, $k_{act}$ significantly decreased (CaV1.3A: 7.4 ± 0.2; n = 9, p < 0.001; Table 1), and $V_{0.5}$,act significantly shifted to more hyperpolarized voltages. Like in CaV1.3A, $I_{Ca}$ inactivation was faster, and CDI was more pronounced, and $I_{Ba}$ was slowed (for all data and statistics see Tables 1–3).

To prove the modulatory role of the last 158 amino acid residues, we tested the capability of this C-terminal fragment to restore the gating changes induced by the truncation. Therefore, GFP-fused peptide (GFP-C158) was expressed together with the respective truncated channel. GFP-C158 reverted the gating changes induced by the truncation.
**CaV1.3 C-terminal Gating Modulation**

To further restrict the protein domain important for CTM activity, we introduced additional truncations in the distal C terminus (Figs. 4–6). Deletion of the last 76 (CaV1.3ΔC76) amino acids induced the same gating effects as described for CaV1.3ΔC158. However, CaV1.3ΔC76 channel activity was not reverted to CaV1.3Δ2-like behavior by co-expression of peptide GFP-C76 (for data and statistics see Tables 1–3). This could not be explained by a lack of GFP-C76 expression because full-length expression of this peptide was confirmed in Western blot experiments where the peptide expressed at comparable levels to GFP-C158 (n = 3, not illustrated). Our data therefore suggest that the last 76 amino acids are required for the formation of CTM activity but cannot functionally recombine with its truncated counterpart to form a modulatory structure. Sequence alignment with other LTCCs (Fig. 6) revealed that truncation ΔC76 cuts in half the most distal highly conserved domain. This may prevent recombination to a functional domain upon co-expression. Hence, we generated CaV1.3ΔC116 in which the conserved region was fully removed and subsequently co-expressed as a single peptide. As shown in Fig. 4 and Tables 1–3, CaV1.3ΔC116 again exhibited functional ICa and Ibα properties similar to CaV1.3Δ2A. However, unlike in CaV1.3ΔC76, the gating properties were reversed to those of CaV1.3Δ2 after co-expression of the GFP-C16 peptide (Fig. 4 and Tables 1–3). Co-expression of the corresponding distal peptides not only reversed activation parameters and CDI but also significantly decreased mean ICa density by 53–63% (p < 0.05, n = 7–11). A similar trend was also seen for Ibα (41–67%; n = 7–11). These data implicate that CaV1.3 α1 subunits contain critical structural elements required for CTM function within the last 76 amino acids and that the last 116 residues perform CTM activity when co-expressed in HEK-293 cells.

**Intramolecular Protein-Protein Interaction Regulates CaV1.3 Channel Gating**—We (15) and others (16) showed that in CaV1.4 α1 subunits the distal CTM interacts with a more proximal domain containing the EF-hand, pre-IQ, and IQ-motif. If such a mechanism also holds true for CaV1.3 channels, CTM-containing peptides should also modulate CaV1.3Δ2A (containing EF-PrelQ-IQ). Interestingly, co-expression of GFP-C158 neither reversed the CaV1.3Δ2A-induced hyperpolarizing shift of the activation range of ICa (CaV1.3Δ2A + GFP-C158: V0.5,act = -12.6 ± 1.5 mV, n = 7; p < 0.001; see Fig. 5a and Table 1) and Ibα (Table 2) nor the voltage-dependent inactivation and CDI (Fig. 5, b and c; Table 3). This prompted us to extend the CaV1.3 Δ1 subunit C terminus to residue 1664 thereby including a highly conserved domain downstream of the IQ-motif that is missing in CaV1.3Δ2A and also contains the so-called PCRD domain previously identified as binding partner for C-terminal peptides in CaV1.2 channels (Fig. 6) (17). As expected, the resulting CaV1.3ΔC473 channels showed CaV1.3Δ2A-like gating (Tables 1–3) but, in contrast to CaV1.3Δ2A, co-expression of GFP-C116 restored CDI (Fig. 5, h and i; Table 3) and the activation and inactivation parameters for ICa (Fig. 5, d–f and h and i; and Tables 1 and 3) and Ibα (Tables 2 and 3) close to CaV1.3Δ2A values. These data provide functional evidence for an important role of proximal residues 1626–1664 and the distal 116 amino acids for CTM function.

To provide further evidence for this mechanism, we measured FRET between different YFP-labeled C-terminal fragments and GFP-labeled C158 (CFP-C158) as well as GFP-labeled C116 (CFP-C116). The construct corresponding to the C-terminal fragment of CaV1.3Δ2A (YFP-EF-PrelQ-IQ) did not show significant FRET with the GFP-C158 probe (legend to Fig. 7). By extending the CaV1.3 YFP-EF-PrelQ-IQ bait to residues 1664 yielding YFP-EF-PrelQ-IQ-PCRD, a strong and significant FRET signal was measured with CFP-C158 as well CFP-C116 (Fig. 7), which is in excellent agreement with our electrophysiological data. Accordingly, no FRET was observed when CFP-C76 peptide was used as a probe (not shown). For CaV1.2
null
significant expression of exon 42A was also found. In whole-brain preparations, exon 42A was 11.0 ± 0.6% (n = 8) of the two mRNAs. Variable expression was found in brain sub-regions with the highest expression in cerebellum (17.1 ± 2.5%, n = 4) and the nucleus accumbens (13.3 ± 1.5%, n = 3) and the lowest in olfactory bulb (2.1 ± 0.2%, n = 3). These data suggest that the short CaV1.3 splice variant can contribute significantly to overall CaV1.3 activity. Notably, our experiments do not rule out the possibility of other short isoforms with gating properties similar to CaV1.342A. Therefore the contribution of short forms to overall CaV1.3 expression is likely to be underestimated by our experiments.

**DISCUSSION**

Here we present functional evidence for a C-terminal automodulatory domain that controls voltage- and Ca²⁺-dependent gating properties of CaV1.3 LTCCs. Such modulation has not been described for CaV1.3 channels before. The absence of the CTM in the short splice variant CaV1.342A provides functional diversity to CaV1.3 channels. CaV1.342A mRNA is expressed together with CaV1.342 in different mouse and human tissues suggesting that this functional diversity is of physiological relevance to adapt CaV1.3 channel activity for specific physiological needs. Activation at negative voltages is one hallmark of CaV1.3 channels (for review see Ref. 6) crucial for adequate neurotransmitter release in IHCs and pacemaker function in the SAN, atrioventricular conduction (30, 31), and neuronal excitability (7, 32). In the absence of the CTM, \( V_{0.5,act} \) is shifted about 10 mV to more negative voltages by decreasing the slope factor of the activation curve but without measurable effects on the activation threshold. This effect was not correlated with current densities, was independent from cell passage number, and was reproducibly observed for splice variant CaV1.342A as well as for several truncations mutants. Moreover, it could not be attributed to artificial manipulations of the CaV1.3α1 subunits because it was observed in wild-type channel constructs not modified by fusion to GFP or introduction of...
other tags. In addition, co-expression of peptides containing the CTM with truncation mutants CaV1.3ΔC116, CaV1.3ΔC158H and CaV1.3ΔC473 reversed this shift. Cells may therefore adjust the activity of CaV1.3-mediated signaling by varying the relative abundance of CTM-containing splice variants.

The functional properties of CaV1.3 and CaV1.3A channels have been studied in previous reports. In two studies functional differences were observed between these splice variants, termed CaV1.3a and CaV1.3b therein, respectively. Calin-Jageman et al. (39) found that VDF of rat CaV1.3 channels expressed in HEK-293T cells was significantly more pronounced for CaV1.3A than for CaV1.3 suggesting an autoinhibitory effect of the C-terminal tail on VDF. Comparisons of I-V relationship and I_Ca were not reported in this study. Zhang et al. (37) expressed hemagglutinin-tagged splice variants in Xenopus laevis oocytes and found that I_Ba of the CaV1.3A construct was not only larger in amplitude but also its I-V relationship was shifted toward more negative voltages, similar to our findings. I_Ca was not reported. Although a more detailed comparison of the biophysical properties of CaV1.3 and CaV1.3A was not the purpose of these studies, they support a modulatory role of the C terminus. In contrast, other groups using the corresponding (5, 13) or different (33) rat CaV1.3 and CaV1.3A/H251 subunit analogues found neither major differences in the activation voltage range nor in CDI (13). Therefore, V_{0.5,act} and V_{max} values for I_Ba as well as the extent of CDI closely resemble our human CaV1.3A channels (5, 13, 12). Even though at present the molecular basis for this difference is unclear, chimeric constructs between rat and human CaV1.3 constructs expressed under identical experimental conditions (e.g. to exclude confounding effects of the heterologous expression system.

FIGURE 7. FRET analysis of the binding of peptide C158 or C116 to various fragments of the CaV1.3 C terminus. N_{FRET} values obtained from co-expression of CFP-C158 or CFP-C116 (probe) with the indicated YFP-tagged CaV1.3 C-terminal fragments in HEK-293 cells. All constructs showed a homogeneous intracellular distribution. As controls, N_{FRET} values are given for CFP co-expressed with YFP-EF-PreIQ-IQ-PCRD (CFP control) and YFP with probes CFP-C158 (YFP control 1) and CFP-C116 (YFP control 2). Controls are not significantly different from zero (one-sample t test against 0). Interaction between fragments was considered when significant difference to both the CFP- and the corresponding YFP control was observed (***, p < 0.001, one-way ANOVA followed by Bonferroni post-test).

FIGURE 8. Tissue expression of CaV1.3 C-terminal splice variants. Qualitative RT-PCR experiments showing expression of both long (containing exon (Ex) 42) and short (containing exon 42A) CaV1.3 isoforms in different mouse (a) and human (b) tissues. GAPDH was used as a housekeeping gene. One representative out of at least three independent experiments is shown. Quantitative Taqman RT-PCR experiments in (c), show the relative expression of exons 42 and 42A in percent of total signal in mouse whole-brain and several brain subregions (***, p < 0.001, unpaired Students t test, all significantly different from 0, one sample t test). Data are given as means ± S.E.
and/or accessory subunit composition) will help to clarify this question.

Our observation of a modulatory C-terminal domain in CaV1.3 adds to recent discoveries of a similar regulatory principle in other LTCC isoforms (see Refs. 15, 17, and references therein). Autoinhibitory control of CaV1.2 channel function was proposed to be based on a binding interaction between a pair of exposed arginine residues and negatively charged residues in α-helical motifs in a proximal (PCRD) and a distal (DCRD) conserved region of the C terminus, respectively (17). In our human CaV1.3 channels, 39 residues (1626–1664) comprising the proximal conserved domain containing the PCRD were necessary to confer modulation, as well as FRET to co-expressed CTM-containing peptides. Neutralization of the two conserved positive charges in PCRD did not prevent FRET implying other essential motifs within this 39-residue PCRD domain. Evidence for a role of DCRD comes from the finding that two conserved negative charges are required to support binding of CFP–C116 to the upstream domain.

The functional consequences of LTCC C-terminal modulation appear different in CaV1.2 and CaV1.3 channels. In CaV1.2, C-terminal truncation does not induce a more negative V0.5,act, but overexpression of the distal C terminus shifts the activation range to more positive voltages and reduces the coupling efficiency of voltage-sensing to channel opening (17). This is in contrast to CaV1.4 and CaV1.3 channels in which removal of the CTM facilitates activation within a more negative voltage range and CTM co-expression restores gating (15 and this paper).

Differences between LTCCs also exist with respect to the modulation of CDI. Although in both channels the CTM is able to interfere with CaM association, the CTM completely prevents CDI in CaV1.4 (15) but only moderates CDI in CaV1.3. To our knowledge the effects of the C-terminal autoinhibitory domain on CDI of CaV1.2 channels has not been systematically analyzed so far.

Our finding of C-terminal modulation of CaV1.3 also raises the important question about potential post-translational proteolytic cleavage in the long C terminus. Such has been reported for CaV1.1 and CaV1.2 channels (18, 34). The CaV1.2 cleavage product not only serves as a potent autoinhibitor when it remains noncovalently bound to the channel (17) but may also dissociate from the α1 subunit thereby serving as a transcriptional regulator after translocation to the nucleus (19). So far biochemical evidence for CaV1.3 C-terminal cleavage is lacking, and it does not appear to function as a transcriptional regulator (19). Instead, as demonstrated here, alternative splicing can generate short and functionally distinct CaV1.3 variants. Such splicing has not yet been reported for CaV1.1, CaV1.2, or CaV1.4 channels.

Our data predict that the expression of CaV1.342A would allow a cell to promote Ca2+ entry through CaV1.3 channels at sub-threshold voltages as predicted from the negative shift of the window current (Fig. 2e). Stronger activation at more negative voltages may also facilitate the onset of upstate potentials in neurons. However, during maintained depolarization, the faster CDI would limit Ca2+ entry through these channels as relevant in neurons with CaV1.3-dependent pacemaking, e.g. dopamine-containing neurons in the substantia nigra (36).

Whereas negative activation of an even small CaV1.3 current could trigger pacemaking, faster CDI would limit Ca2+ entry during ensuing action potentials. This may be important in these neurons that are susceptible to Ca2+ toxicity and neurodegeneration in Parkinson disease (36). In contrast, the CTM in CaV1.342 channels may be required for longer lasting Ca2+ signals triggered by stronger depolarization inducing cAMP-response element-binding protein phosphorylation and synaptic plasticity (37).

Our study raises several important questions that need to be addressed in future studies. The fact that the increase in current density observed in CaV1.342A channels seems not to be due to increased expression density (and increased gating currents, see Fig. 2) suggests differences in the unitary current (e.g. increase in single-channel conductance or open probability or prolonged open times). Single-channel analysis will also be required to provide a mechanistic explanation for the shift in activation gating parameters in the short CaV1.3 channels. Changes in the unitary conductance of CaV1.3 channels induced by structural alterations within the CaM interaction domains in the C terminus well outside the known pore-forming regions have been described (38) providing evidence for a conformational link of the CaM binding domain not only with the gating machinery but also with the channel pore.

Several proteins binding to interaction domains of the C terminus have been described. For example, in neurons the multifunctional PDZ protein Erbin binds to a PDZ-binding domain formed by the last four amino acid residues of CaV1.342 and thereby relieves the autoinhibitory effect of the C terminus on VDF (see Ref. 39 and see above). Also Shank protein (40) and RIM-binding protein (41) are predicted to bind selectively to the C terminus of CaV1.3 channels and may therefore affect CTM function. The fact that the distal C terminus comprising the CTM also contains regulatory sites, including sites for cAMP-dependent protein kinase phosphorylation (42) and AKAP-15 (43), raises the interesting possibility that their interference with the activity of the CTM itself could also serve as mechanism to fine-tune CaV1.3 function. Such a mechanism has recently been proposed to explain the still enigmatic mechanism of cAMP-dependent protein kinase-dependent activation of CaV1.2 LTCCs (35).

Taken together, we have identified a modulatory domain in CaV1.3 LTCCs responsible for profound differences in the Ca2+- and voltage-dependent gating of the different C-terminal splice variants. Given that many unique physiological functions of CaV1.3 depend on the negative activation range of the channel and the amount of Ca2+ ions entering during plateau (7) or single action potentials (8), our findings identify the CTM and factors that modify its activity (such as alternative splicing) as elements suitable to determine electrical excitability. Future experiments must also address the interesting possibility that interference with this C-terminal regulatory mechanism can also be exploited for pharmacological intervention, as an alternative to classical Ca2+ channel modulators.
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