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To cite this version:

Mitsuru Hirano, Yoshinori Takada, Chee Fah Wong, Kazuma Yamaguchi, Hiroshi Kotani, et al.. C-terminal splice variants of P/Q-type Ca(2+) channel CaV2.1 α1 subunits are differentially regulated by Rab3-interacting molecule proteins. Journal of Biological Chemistry, American Society for Biochemistry and Molecular Biology, 2017, 292 (22), pp.9365–9381. 10.1074/jbc.M117.778829. hal-01832955

HAL Id: hal-01832955
https://hal.archives-ouvertes.fr/hal-01832955
Submitted on 13 Jul 2018

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C-terminal splice variants of P/Q-type Ca^{2+} channel Ca_{v}2.1 α_{1} subunits are differentially regulated by Rab3-interacting molecule proteins

Received for publication, January 29, 2017, and in revised form, March 26, 2017 Published, Papers in Press, April 4, 2017, DOI 10.1074/jbc.M117.778829

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Edited by F. Anne Stephenson

Voltage-dependent Ca^{2+} channels (VDCCs) mediate neurotransmitter release controlled by presynaptic proteins such as the scaffolding proteins Rab3-interacting molecules (RIMs). RIMs confer sustained activity and anchoring of synaptic vesicles to the VDCCs. Multiple sites on the VDCC α_{1} and β subunits have been reported to mediate the RIMs-VDCC interaction, but their significance is unclear. Because alternative splicing of exons 44 and 47 in the P/Q-type VDCC α_{1} subunit Ca_{v}2.1 gene generates major variants of the Ca_{v}2.1 C-terminal region, known for associating with presynaptic proteins, we focused here on the protein regions encoded by these two exons. Co-immunoprecipitation experiments indicated that the C-terminal domain (CTD) encoded by Ca_{v}2.1 exons 40–47 interacts with the α-RIMs, RIM1α and RIM2α, and this interaction was abolished by alternative splicing that deletes the protein regions encoded by exons 44 and 47. Electrophysiological characterization of VDCC currents revealed that the suppressive effect of RIM2α on voltage-dependent inactivation (VDI) was stronger than that of RIM1α for the Ca_{v}2.1 variant containing the region encoded by exons 44 and 47. Importantly, in the Ca_{v}2.1 variant in which exons 44 and 47 were deleted, strong RIM2α-mediated VDI suppression was attenuated to a level comparable with that of RIM1α-mediated VDI suppression, which was unaffected by the exclusion of exons 44 and 47. Studies of deletion mutants of the exon 47 region identified 17 amino acid residues on the C-terminal side of a polyglutamine stretch as being essential for the potentiated VDI suppression characteristic of RIM2α. These results suggest that the interactions of the Ca_{v}2.1 CTD with RIMs enable Ca_{v}2.1 proteins to distinguish α-RIM isoforms in VDI suppression of P/Q-type VDCC currents.

Fine regulation of neurotransmitter release is integral to adaptive functions of the nervous system, including learning, memory, and cognition. Neurotransmitter release is triggered by depolarization-induced Ca^{2+} influx via voltage-dependent Ca^{2+} channels (VDCCs) in presynaptic active zones (AZs), where synaptic vesicles (SVs) dock in close vicinity to VDCCs at the presynaptic membrane (1, 2). Among different VDCC types, which are distinguished on the basis of their pharmacological and biophysical properties, L-, N-, R-, and P/Q-types have been reported to mediate Ca^{2+} influx responsible for neurotransmitter release (3–6). Different VDCC types show distinct tissue expression patterns, subcellular localizations, activity-dependent properties, and amounts of Ca^{2+} influx, all of which contribute to the fine regulation of neurotransmitter release (7–18). In particular, the local Ca^{2+} concentration ([Ca^{2+}]_{local}) and spacing between VDCCs and SVs are tightly regulated by the molecular organization of presynaptic AZs and influence the dynamic properties of neurotransmitter release (2, 9, 18–27). It is also understood that the number of open VDCCs, which determines the [Ca^{2+}]_{local} and release probability of SVs, depends on the efficiency of targeting and availability of VDCCs in the AZ (28). In response to membrane depolarization, VDCCs open to evoke [Ca^{2+}]_{local} rise and simultaneously close via inactivation. This negative feedback reduces the number of VDCCs available and restricts the amplitude of Ca^{2+} influx, which is important for the diversification of Ca^{2+} signaling (29). Inactivation of VDCCs in the presynapse is largely dependent upon the inward Ca^{2+} current magnitude and displays only a weak voltage dependence (13, 30).

In the P/Q-type, VDCCs are composed of the pore-forming α_{1} subunit (Ca_{v}2.1) and accessory α_{2}/δ, β, and γ subunits. Ca_{v}2.1 is the most abundantly expressed VDCC α_{1} subunit in...
the mammalian brain (31), and mutations in the CaV2.1 gene, caca1a, cause several autosomal-dominant neurological disorders, including familial hemiplegic migraine type 1, episodic ataxia type 2, and spinocerebellar ataxia type 6 (SCA6) (32–34). Multiple functional P/Q- and N-type VDCC variants are generated by alternative splicing of subunit genes (31, 33), different subunit compositions (36), post-translational processing (37), and association with interacting proteins (20, 24, 38, 39). Several types of P/Q-type VDCC complexes can be co-localized in a single neuron and are believed to contribute to the fine-tuning of neuronal processes, such as neurotransmitter release, because formation of each type of CaV2.1 channel complex is regulated in a different manner (35, 40–42).

Rab3-interacting molecules (RIMs) are multidomain scaffolding proteins expressed in secretory cells (43). Long isoform α-RIMs, including RIM1α and RIM2α, contain an N-terminal zinc finger domain, a central PDZ domain, and two C-terminal domains, C2A and C2B. Physiological experiments have shown that α-RIMs are essential for docking and priming of SVs and for recruiting and tethering VDCCs to the presynaptic AZ, thereby regulating VDCC function and short-term plasticity of neurotransmitter release (20, 22, 44–49). We have reported that α-RIMs increase neurotransmitter release by sustaining Ca2+ influx through strong inhibition of voltage-dependent inactivation (VDI) of VDCCs and by anchoring vesicles in the vicinity of VDCCs via interaction with VDCC β subunits (Fig. 1A) (20). We have also revealed that the RIM C-terminal C2B domain is essential for RIM-β subunit interaction and inhibition of VDI of VDCCs (20, 39). Mutations in the gene encoding RIMs associated with autism and cone-rod dystrophy, CORD7, modify this interaction and/or the regulation of VDCC currents (50, 51). Functional coupling of RIM1α to β subunits of VDCCs is also essential for insulin secretion in non-neuronal cells (52). In addition to the β subunits, the PDZ domain of α-RIMs has been reported to interact with the PDZ-binding motif located at the end of the C terminus of α1 subunits to modulate localization of P/Q- and N-type VDCC complexes to presynaptic AZs (Fig. 1A) (22). Thus, α-RIMs may interact with VDCC complexes through multiple sites of the constituent subunits. However, the significance of multipoint interactions among VDCC α1 subunits, β subunits, and α-RIMs, as well as functional effects of interactions between α-RIMs and the CaV2.1 C terminus on VDCC currents, remains unclear.

To quantify the functional significance of multipoint interaction, it is interesting to focus on alternative splicing of exons 44 and 47, because this generates major CaV2.1 C-terminal splice variants expressed in the human cerebellum (Fig. 1B and Table 1). Introns 42–44 are flanked by GT/AG splice-site sequences, and alternative splicing leads to either the inclusion or exclusion of exons 43 and 44 (referred to as (+ 43 or – 43) and (+ 44 or – 44) in Fig. 1B) (53). Insertion of a pentanucleotide GCCAG at the beginning of exon 47 allows in-frame translation of exon 47 to produce a long version of the C terminus (referred to as 47) in Fig. 1B). Otherwise, omission of the GCCAG in transcripts causes a frameshift, leading to stop codon termination near the beginning of exon 47 (referred to as Δ47 in Fig. 1B) to generate the human homolog of the rabbit VDCC α1A subunit BI-1 (Fig. 1B) (31, 34, 53). The 12-amino acid region encoded by exon 44 starts with the arginine residue, which is located 292 amino acids downstream from the transmembrane segment S6 of repeat IV. The exon 44-encoded region is thought to have an AT-hook domain, which is a tripartite DNA-binding motif specific for AT-rich sequences that is typically found in nuclear proteins and DNA-binding proteins (54, 55). The 244-amino acid region encoded by exon 47 with the GCCAG insertion starts with the glycine residue, which is located 451 amino acids downstream from S6 of repeat IV. The exon 47-encoded region has Src homology 3 and PDZ domain-binding motifs, which are targets of synaptic proteins such as CASK, Mint1, RIM-binding protein (RIM-BP), and α-RIMs (19, 22, 56). It is also known that expansion of the polyglutamine tract (polyQ), encoded by CAG trinucleotide repeats in exon 47 of human CaV2.1, causes the neurological disease, SCA6 (34).

Here, we studied the interactions between α-RIMs and the CaV2.1 C-terminal regions encoded by exons 44 and 47. We revealed the functional impacts of α-RIM interaction with different CaV2.1 C-terminal regions on its VDI. The 17 amino acid residues on the C-terminal side of the polyQ stretch play an essential role in the pronounced VDI suppression characteristic of RIM2α. In the CaV2.1 splice variant lacking exons 44 and 47, VDI suppression remained intact for RIM1α, but for RIM2α, it was reduced to a level comparable with that of RIM1α. These results suggest that the CTD region plays an important role in the α-RIM isoform-dependent potentiation of VDI suppression. Also, our data reveal that the interaction of α-RIMs with the CTD regions encoded by exons 44 and 47 is not essential for the suppressive effects of α-RIMs on VDI, further raising the possibility that interactions of the VDCC β subunits with α-RIMs underlie their strong suppressive effects on the VDI of VDCCs.

Results

Characterization of CaV2.1 C-terminal splice variation in the human cerebellum

We have previously demonstrated that the RIM C-terminal region containing the C2B domain interacts with VDCC β subunits (20, 39, 50). It has also been reported that PDZ domains of α-RIMs interact with the PDZ-binding motif located at the C-terminal end of CaV2.1 and CaV2.2 (Fig. 1A) (22, 57). The C-terminal region is highly divergent in VDCC α1 subunits because of multiple alternative splice sites (58). In particular, splicing out exon 47 generates CaV2.1 splice variants that lack the most C-terminal region, including the PDZ-binding motif. It is important to quantitatively assess the relative significance of α-RIM interactions with the C-terminal region of CaV2.1 and the VDCC β subunit by comparing α-RIM actions on P/Q-type VDCCs containing different CaV2.1 splice variants carrying the C terminus with and without the α-RIM-interacting region. Previous studies have revealed that exons 43, 44, and 47 contribute to C-terminal splice variations in human CaV2.1 (53), but relative levels of splice variants with different combinations of these exons have not been quantified. We performed sequence analysis of PCR products from a cDNA library of the human cerebellum, in which abundant expressions of α-RIMs and CaV2.1 mRNAs were reported (39, 59, 60). A set of PCR oligonucleotide primers were located in exon 42 (forward) and...
exon 47 (reverse). Agarose gel electrophoresis (1%) revealed a broad band of PCR products of \(\sim 1000\) bp consistent with the predicted sizes ranging from 834 to 989 bp (Fig. 1C). This DNA band was subcloned into a vector, and the relative levels of splice variants were determined by counting the number of clones containing each exon. The relative proportions of individual splice variants of exon +43, -43, +44, -44, and 47/Δ47 were detected at the highest relative proportion (56%) (Fig. 1A and Table 1). Relative proportions of CaV2.1 (Δ44,47), CaV2.1 (Δ44,47) CaV2.1 (Δ44,47) were 30, 11, and 3%, respectively (Fig. 1A and Table 1).

Interaction between α-RIMs and CaV2.1 C-terminal splice variants

We next performed yeast two-hybrid screening of a human brain cDNA library using the C-terminal domain encoded by exons 40–47 of human CaV2.1, CaV2.1 CTD (Δ44,47), as bait, and we identified an interaction between CaV2.1 CTD and the amino acid residues 487–1349 of human RIM2α (GenBank™ accession number NM_001127221) (Fig. 2A). We also performed co-immunoprecipitation (co-IP) experiments to confirm RIM-CaV2.1 CTD interactions (Fig. 2B). As a control, we chose the VDCC β4 subunit, because β4 is abundantly expressed in the brain and the spontaneous β4 mutant lethalic mouse (cacnb4<sup>−/−</sup>) has clear neurological defects, supporting the physiological significance of β4 in the brain (61, 62). YFP-tagged β4 was co-immunoprecipitated with FLAG-tagged α-RIMs, RIM1α and RIM2α, in HEK293T cells (Fig. 2C), as reported previously (20, 39). Next, we performed co-IP between YFP-tagged α-RIMs and FLAG-tagged CTDs of CaV2.1 variants derived from alternative splicing of exons 44 and 47 in HEK293T cells. RIM1α and RIM2α were co-immunoprecipitated with the CaV2.1 CTD splice variants except for the variant lacking exons 44 and 47 (Fig. 2D). These results suggest that the two regions encoded by exons 44 and 47 contribute significantly to the interaction between the CaV2.1 CTD and α-RIMs.

### Table 1

Characterization of CaV2.1 C-terminal splice variation in the human cerebellum

| Splice variant | % of total | n |
|---------------|------------|---|
| -43           | 0          | 87 |
| +43           | 100        |   |
| -44           | 14         | 87 |
| +44           | 86         |   |
| 47            | 66         | 85 |
| +43, +44,47   | 56         | 80 |
| +43, -44,47   | 11         |   |
| +43, +44,Δ47  | 30         |   |
| +43, -44,Δ47  | 3          |   |

n means number of clones sequenced.
We have previously reported that RIMs strongly suppress VDIs of neuronal VDCCs by interacting with the β subunits (20, 39, 50). It has also been reported that the RIM-CaV2.1 C-terminal interaction modulates localization of VDCCs to presynaptic AZs (22). However, the effects of RIM-CaV2.1 C-terminal interaction on VDCC properties have not been examined. We characterized whole-cell Ba²⁺ currents through recombinant P/Q-type VDCCs containing the CaV2.1 splice variants, β₄ and α₂δ₁ subunits, in HEK293 cells. We chose Ba²⁺ as a charge carrier, because CaV₂.1 splice variants show different Ca²⁺-dependent properties in HEK293 cells (63). Voltage dependence of inactivation at different voltages (inactivation curve) (Fig. 3A) was first examined in CaV₂.1 (+44,47)-expressing and CaV₂.1 (-44,-47)-expressing cells. No significant difference was detected between inactivation curves of CaV₂.1 (+44,47) and CaV₂.1 (-44,-47) channels (Fig. 3B and Table 2), which is consistent with previous reports (63, 64). In cells co-expressing α-RIMs and CaV₂.1 (-44,-47), we detected remarkable inactivation curve shifts toward depolarizing potentials, as described previously (20, 39). Co-expression of RIM2α induced a more significant inactivation curve shift toward depolarizing potentials compared with that for RIM1α in CaV₂.1 (+44,47)-expressing cells, whereas this difference between α-RIMs was not observed in CaV₂.1 (-44,-47)-expressing cells (the half-in-
activation potentials \( (V_{0.5}) \) for Ca\(_{2.1} (+44,47) \) and Ca\(_{2.1} (-44,47) \) with RIM2\( \alpha \) were \(-6.7 \pm 2.3 \) and \(-17.2 \pm 2.1 \) mV, respectively (Fig. 3B and Table 2). Considering that the previously reported PDZ domain-binding region is not present in the C-terminal end of Ca\(_{2.1} (-44,47) \), RIM-\( \beta \) subunit interaction may play major roles in suppressing VDI of VDCCs, whereas RIM-Ca\(_{2.1} \) C-terminal interaction potentiates this regulatory effect exerted by \( \alpha \)-RIMs on VDCCs.

To further assess the importance of interactions via the regions encoded by exons 44 and 47 in this potentiation characteristic of RIM2\( \alpha \) in suppression of VDI, we examined the effect of RIM2\( \alpha \) on Ca\(_{2.1} (-44,47) \) and Ca\(_{2.1} (+44,47) \) inactivation curves. A significantly enhanced shift in the inactivation curve toward depolarizing potentials was detected in cells co-expressing RIM2\( \alpha \) and Ca\(_{2.1} (-44,47) \), but not in cells co-expressing RIM2\( \alpha \) and Ca\(_{2.1} (+44,47) \), compared with cells co-expressing RIM2\( \alpha \) and Ca\(_{2.1} (-44,47) \) \( (V_{0.5} \) of Ca\(_{2.1} (-44,47) \) and Ca\(_{2.1} (+44,47) \) with RIM2\( \alpha \) was \(-8.6 \pm 2.0 \) and \(-15.4 \pm 2.4 \) mV, respectively) (Fig. 3B and Table 2). We could not observe a significant difference between Ca\(_{2.1} (+44,47) \) and Ca\(_{2.1} (-44,47) \) in current density-voltage \( (I-V) \) relationships, with or without RIM2\( \alpha \) co-expression (Fig. 4 and Table 3). These results suggest that the RIM-Ca\(_{2.1} \) C-terminal interaction via the region encoded by exon 47 is important for the potentiated suppressive effect of RIM2\( \alpha \) on VDI.

Inactivation kinetics of P/Q-type VDCCs was characterized by analyzing the decay phase of Ba\(^{2+} \) currents evoked by 1-s test pulses in HEK293 cells (Fig. 5A). The decay phase was well fitted by two exponential functions with a non-inactivating component (Fig. 5B) as reported previously (41). The two exponential time constants \( (\tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) ) and the ratio of fast, slow, and non-inactivating components were similar in Ca\(_{2.1} (+44,47) \)- and Ca\(_{2.1} (-44,47) \)-expressing cells at test poten-
α-RIMs diversify inactivation of Ca_{v}2.1 splice variants

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Figure 4. Effects of α-RIMs on the I-V relationships of P/Q-type Ca_{v}2.1 channels. I-V relationships of Ca_{v}2.1 splice variants in HEK293 cells expressing β_{3a} and α_{Qσ-1} subunits. Left, representative traces for Ba^{2+} currents on application of test pluses from -40 to 60 mV with 10-mV increments from a holding potential \( V_{h} \) of -90 mV. Right, I-V relationships of Ca_{v}2.1 splice variants. See Table 3 for statistical significance of the differences. Error bars, S.E.

Table 3
Effect of α-RIMs on the I-V relationships of P/Q-type VDCCs in HEK293 cells expressing Ca_{v}2.1, α_{Qσ-1}, and β_{3a}.

| Current density \( \mu A/picoFarad \) | \( V_{h} \) \( mV \) | \( k \) \( mV \) |
|----------------------------------------|----------------|-------------|
| Ca_{v}2.1 (+44,47) Vector              | -7.1 \± 10.8 (5) | -3.0 \± 1.3 (5) | 4.5 \± 0.2 (5) |
| Ca_{v}2.1 (+44,47) RIM1a              | -97.5 \± 21.3 (5) | -5.1 \± 1.9 (5) | 4.5 \± 0.4 (5) |
| Ca_{v}2.1 (+44,47) RIM2a              | -83.1 \± 7.5 (7) | -3.7 \± 1.1 (7) | 3.7 \± 0.6 (7) |
| Ca_{v}2.1 (-44,Δ47) Vector            | -111.5 \± 13.8 (4) | -4.0 \± 1.6 (4) | 3.0 \± 0.3 (4) |
| Ca_{v}2.1 (-44,Δ47) RIM1a             | -123.8 \± 30.6 (4) | -3.2 \± 1.7 (4) | 3.6 \± 0.6 (4) |
| Ca_{v}2.1 (-44,Δ47) RIM2a             | -102.5 \± 7.4 (7) | -6.5 \± 1.0 (7) | 3.6 \± 0.3 (7) |

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It is important to note that, without the interaction of α-RIMs, these splice variants were indistinguishable in channel properties such as inactivation curve (Fig. 3 and Table 2), inactivation kinetics (Fig. 5 and Table 4), and I-V relationship (Fig. 4 and Table 3). This underscores the significance of protein-protein interaction in differentiating functional properties of alternatively spliced Ca_{v}2.1 variants.

Biochemical characterization of the RIM2α-Ca_{v}2.1 C-terminal interaction

The Ca_{v}2.1 mutant, Ca_{v}2.1 (+44,47) ΔDDWC, which lacks four amino acids (DDWC) in the C-terminal end was constructed, because these four amino acids bind to the PDZ domain of α-RIMs (22, 57). Inactivation curves of whole-cell Ba^{2+} currents in HEK293 cells co-expressing RIM2α and Ca_{v}2.1 (+44,47) ΔDDWC were indistinguishable from those in cells co-expressing RIM2α and Ca_{v}2.1 (+44,47), showing shifts toward depolarizing potentials compared with the inactivation curves in cells co-expressing RIM2α and Ca_{v}2.1 (-44,Δ47) \( V_{0.5} \) of Ca_{v}2.1 (+44,47) ΔDDWC, Ca_{v}2.1 (+44,47), and Ca_{v}2.1 (-44,Δ47) were \(-6.7 \pm 3.2 \) mV, \(-6.7 \pm 2.3 \) mV, and \(-17.2 \± 2.1 \) mV, respectively (Fig. 6A and Table 5). In co-IP experiments, YFP-tagged RIM2α showed association with FLAG-tagged Ca_{v}2.1 CTD (+44,47) ΔDDWC in HEK293T cells (Fig. 6B). The intensity of the co-IP band for RIM2α normalized to the IP band for the CTD was significantly decreased in cells co-expressing RIM2α and Ca_{v}2.1 CTD (+44,47) ΔDDWC, compared with cells co-expressing RIM2α and Ca_{v}2.1 CTD (+44,47) (Fig. 6C). These data suggest that DDWC in the region encoded by exon 47 contributes to the RIM2α-Ca_{v}2.1 C-terminal interaction, as reported previously (22, 57), but not to potentiation of VDI suppression by RIM2α.

To clarify the RIM2α-interacting regions responsible for the potentiation of VDI suppression by RIM2α, additional deletion mutants of Ca_{v}2.1 were constructed (Fig. 7A). In cells...
co-expressing RIM2α with CaV2.1 (+44,2364X) or CaV2.1 (+44,2344X), in which C-terminal residues 2365–2505 or 2345–2505 were deleted, respectively, the inactivation curves were similar to those in cells co-expressing RIM2α with CaV2.1 (+44,47). In contrast, in cells co-expressing RIM2α with CaV2.1 (+44,2327X) or CaV2.1 (+44,2313X), in which C-terminal residues 2328–2505 or 2314–2505 were deleted, respectively, the inactivation curves were similar to those in cells co-expressing RIM2α and CaV2.1 (+44,Δ47) (Fig. 7B and Table 5).

These data suggest that 17 amino acid residues, 2328–2344 (RPGRAATSGPRRYPGPT), on the C-terminal side of the polyQ stretch of CaV2.1 (+44,47) is a RIM2α-interacting region that potentiates the suppressive effect on VDI.

In co-IP experiments, however, YFP-tagged RIM2α showed a comparable level of co-IP with FLAG-tagged CTD of CaV2.1 (+44,2344X) and FLAG-tagged CTD of CaV2.1 (+44,2327X) but a decreased level of co-IP with CTD of CaV2.1 (+44,2275X) (Fig. 7C). To eliminate possible contributions of exon 44-en-
α-RIMs diversify inactivation of Ca_{2.1} splice variants

Table 4

| Effect of α-RIMs on inactivation kinetics of P/Q-type VDCCs in HEK293 cells expressing Ca_{2.1}s, α_{β₁}, and β_{4} |
|---|
| * is p < 0.05; ** is p < 0.01; *** is p < 0.001 versus vector. # is p < 0.05; ## is p < 0.01 versus RIM1α. † is p < 0.05; †† is p < 0.01 versus Ca_{2.1} (−44,Δ47) for cells co-expressing RIM2α. The number of cells analyzed are indicated in parentheses. |

| Fraction of components | Time constants |
|---|---|
| mV | Fast τ_{fast} | Slow τ_{slow} | Sustained |
| Ca_{2.1} (−44,Δ47) Vector (8) | 0.84 ± 0.05 | 0.84 ± 0.05 | 0.84 ± 0.05 | 0.84 ± 0.05 |
| Ca_{2.1} (−44,Δ47) RIM1α (11) | 0.41 ± 0.04*** | 0.38 ± 0.03 | 0.32 ± 0.02*** | 0.32 ± 0.02*** |
| Ca_{2.1} (−44,Δ47) RIM2α (11) | 0.20 ± 0.03**, # | 0.28 ± 0.05 | 0.53 ± 0.06**, †† | 0.53 ± 0.06**, †† |
| Ca_{2.1} (−44,Δ47) DDWC (5) | 0.84 ± 0.05 | 0.84 ± 0.05 | 0.84 ± 0.05 | 0.84 ± 0.05 |
| Ca_{2.1} (−44,Δ47) DDWC, 55°C | 0.30 ± 0.04 | 0.30 ± 0.04 | 0.30 ± 0.04 | 0.30 ± 0.04 |

Figure 6. Effects of RIM2α on VDI of the P/Q-type CaV2.1 (Fig. 7A). Effects of RIM2α on inactivation curves of Ba^{2+} currents mediated by P/Q-type CaV2.1 (−44,47) DDWC, which lacks DDWC at the C-terminal end of CaV2.1 (−44,47), in HEK293 cells expressing β_{4}, β_{1}, and α_{β₁} subunits. The inactivation curve in cells co-expressing RIM2α and CaV2.1 (−44,47) (dashed dark gray line) is taken from Fig. 3 and is shown for comparison. See Table 5 for statistical significance of the differences. Error bars, S.E. B, interaction of FLAG-tagged CaV2.1 CTD (−44,47) with monoclonal anti-FLAG antibody, followed by WB with polyclonal anti-YFP antibody. Input is 10% of the amount of cell lysate used for co-IP and is analyzed by WB using polyclonal anti-YFP antibody. IP of FLAG-tagged CaV2.1 CTDs with monoclonal anti-FLAG antibody is analyzed by WB using polyclonal anti-YFP antibody. C, quantification of the data shown in B. The intensity of the co-IP band for RIM2α normalized to the IP band for the CTD (CaV2.1 CTD (−44,47), 100%; CaV2.1 CTD (−44,47) DDWC, 55°C ± 20%) (92). Data for nine experiments are presented in scatter plots. The vertical error bars represent standard deviations, and the mean values are indicated with horizontal bars. ***, p < 0.001; statistical significance between CTD (−44,47) and CTD (−44,47) DDWC. |

coded amino acid residues to the interaction of RIM2α with CTD mutants, we next constructed deletion mutants of the CTD of CaV2.1 (−44,47). The co-IP was nearly abolished for the CTD of CaV2.1 (−44,2263X), in which 2263X corresponds to 2275X in CaV2.1 (−44,47) (Fig. 7D). This result suggests that the amino acid residues 2264–2280 (GTSTPRGRGRQRPQTPS) of CaV2.1 (−44,47), which correspond to 2276–2292 of CaV2.1 (−44,47), are an important region for the RIM2α–CaV2.1 CTD interaction. However, the co-IP experiments failed to unveil interaction between the 17 amino acid residues, 2328–2344, of CaV2.1 (−44,47) with RIM2α, although this region is supposed to be essential for potentiated VDI suppression by RIM2α.

To demonstrate interaction of the 2328–2344 amino acid residues, contributions of the exon 44 region and residues 2276–2292 of CaV2.1 (−44,47) were eliminated in deletion mutants based upon CaV2.1 (−44,Δ2264–2280). The level of co-IP was markedly diminished for the CTD of CaV2.1 (−44,2264–2280,2315X) and CaV2.1 (−44,Δ2264–2280,2301X) compared with the CTD of CaV2.1 (−44,Δ2264–2280,2323X) and CaV2.1 (−44,2264–2280,2323X) (Fig. 7E). This result suggests that the 17 amino acid residues, 2316–2332, of CaV2.1 (−44,47), which correspond to 2328–2344 of CaV2.1 (−44,47), indeed constitute an important binding region for RIM2α to exert a potentiated suppressive effect on VDI.

It is interesting to note that deletion of the region encoded by exon 44 failed to elicit suppression of interaction of the CaV2.1 CTD containing the region encoded by exon 47 (compare the bands of CaV2.1 CTD (−44,47) and CaV2.1 CTD (−44,47) in Fig. 2D). In contrast, deletion of the region encoded by exon 47 suppressed the interaction of the CaV2.1 CTD containing the region encoded by exon 44 (compare the bands of CaV2.1 CTD (−44,47) and CaV2.1 (−44,47) in Fig. 7C). These results suggest that the region encoded by exon 47 binds more strongly to α-RIMs compared with the region encoded by exon 44. Thus, formation of RIM2α–CaV2.1 complexes is mediated by multi-point interaction.
Table 5
Effects of chimeric RIM and RIM2α deletion mutant on inactivation properties of P/Q-type VDCCs in HEK293 cells expressing CaV2.1, α2δ-1, and β2

| Inactivation parameters | α | V_{0.5} (mV) | k (mV) |
|-------------------------|---|------------|------|
| CaV2.1 (+44,47)         | RIM2α | 0.53 ± 0.04 (13) | -6.7 ± 2.3 (13)** | -9.9 ± 1.0 (13) |
| CaV2.1 (−44,47)         | RIM2α | 0.61 ± 0.02 (15) | -17.2 ± 2.1 (15) | -7.7 ± 0.6 (15) |
| CaV2.1 (+44,47) ΔDDWC | RIM2α | 0.55 ± 0.05 (7) | -6.7 ± 3.2 (7)** | -7.2 ± 1.1 (7) |
| CaV2.1 (+44,47) 2366X  | RIM2α | 0.51 ± 0.07 (6)** | -6.3 ± 2.3 (6)** | -5.7 ± 0.7 (6) |
| CaV2.1 (+44,47) 2344X  | RIM2α | 0.54 ± 0.04 (6) | -8.3 ± 3.0 (6)** | -8.2 ± 0.9 (6) |
| CaV2.1 (+44,47) 2327X  | RIM2α | 0.70 ± 0.03 (6) | -14.1 ± 2.4 (6) | -8.4 ± 0.9 (6) |
| CaV2.1 (+44,47) 2315X  | RIM2α | 0.67 ± 0.04 (8) | -13.3 ± 1.2 (8) | -6.8 ± 0.5 (8) |
| CaV2.1 (+44,47)         | RIM2α (1183–1572) | 0.49 ± 0.07 (7) | -4.7 ± 2.6 (7)** | -8.3 ± 0.7 (7) |
| CaV2.1 (−44,47)         | RIM2α (1183–1572) | 0.43 ± 0.06 (7)** | -1.9 ± 3.2 (7)** | -5.3 ± 1.2 (7) |
| CaV2.1 (−44,47)         | RIM1–2 chimera | 0.59 ± 0.03 (3) | -15.9 ± 3.3 (3) | -7.6 ± 1.5 (3) |
| CaV2.1 (−44,47)         | RIM2α (1183–1572) | 0.69 ± 0.04 (6) | -14.2 ± 4.0 (6) | -7.0 ± 1.4 (6) |

Characterization of the RIM2α region responsible for interaction with the CaV2.1 C terminus

To identify the region in RIM2α that interacts with the CaV2.1 C terminus, we constructed the chimeric RIM, RIM1–2 chimera, with amino acid residues from the N terminus to the C2A domain of RIM1α and from the C2A domain of RIM2α (Fig. 8A). We examined effects of the RIM1–2 chimera on whole-cell Ba2+ currents mediated by CaV2.1 (+44,47) - and CaV2.1 (−44,47)-containing VDCCs (Fig. 8B). Significant enhancement of inactivation curve shift by the RIM1–2 chimera toward depolarizing potentials was observed for CaV2.1 (+44,47) compared with CaV2.1 (−44,47) (V_{0.5} for CaV2.1 (+44,47) and CaV2.1 (−44,47) with RIM1–2 chimera was -4.7 ± 2.6 and -15.9 ± 3.3 mV, respectively (Fig. 8B and Table 5). This tendency of the RIM1–2 chimera suggests that the region on the C-terminal side of the C2A domain is important for potentiating the suppressive effect of RIM2α on VDI. We also tested a mutant of RIM2α(1183–1572) composed of the amino acid residues 1183–1572 of RIM2α corresponding to the β subunit-binding region in RIM1α (residues 1079–1463 of RIM1α) (Figs. 2B and 8A) (20, 39). In cells co-expressing RIM2α(1183–1572) and CaV2.1 (+44,47), we also detected potentiation of the suppressive effect on VDI as observed in cells co-expressing RIM2α(1183–1572) and CaV2.1 (−44,47) (V_{0.5} and the rates of the inactivating component were -1.9 ± 3.2 mV and 0.43 ± 0.06, respectively, for CaV2.1 (+44,47) with RIM2α(1183–1572) and 14.2 ± 4.0 mV and 0.69 ± 0.04, respectively, for CaV2.1 (−44,47) with RIM2α(1183–1572)) (Fig. 8C and Table 5). Associations between YFP-tagged RIM2α (1183–1572) and FLAG-tagged mutants based upon CaV2.1 CTD (−44,Δ2264–2280), which has a deletion of the region corresponding to 2328–2344 of CaV2.1 (+44,47), were tested by co-IP experiments. RIM2α(1183–1572) showed co-IP with the CTD of CaV2.1 (−44,Δ2264–2280, 2332X) but not with the CTD of CaV2.1 (−44,Δ2264–2280, 2315X) or CaV2.1 (−44,Δ2264–2280, 2301X) (Fig. 8D). These data suggest that the C-terminal region containing the C2β domain is critical for RIM2α to potentiate suppression of VDI and binds with 2328–2344 of CaV2.1 (+44,47) in addition to the β subunits (20, 39).

Effects of polyQ elongation in the CaV2.1 C-terminal region on regulation of VDI by RIM2α

The polyQ stretch is an interesting characteristic of the CaV2.1 C-terminal primary structure. SCA6 is caused by expansion of the polyQ tract in the human CaV2.1 gene from a normal repeat size range of 4–17 to a size range of 20–33 (34, 54, 66). To confirm the effect of polyQ expansion on the interaction between the CaV2.1 C terminus and RIM2α, association between YFP-tagged RIM2α and FLAG-tagged CTD of CaV2.1 (+44,47) Gln-40 with an elongated polyQ stretch of 40 residues was tested by co-IP in HEK293T cells (Fig. 9A). The intensity of the co-IP band for RIM2α normalized to the IP band for the CTD was moderately but significantly decreased for CaV2.1 CTD (+44,47) Gln-40 compared with that for CaV2.1 CTD (+44,47) with a polyQ stretch of 11 residues (Fig. 9B). To examine the effect of polyQ elongation on suppression of VDI by RIM2α, a recombinant P/Q-type VDCC was expressed as a complex of CaV2.1 (+44,47) with polyQ expansion (CaV2.1 (+44,47) Gln40), β2, and α2δ-1 subunits in HEK293 cells (Fig. 9C). Without co-expression of α-RIMs, inactivation curves of whole-cell Ba2+ currents elicited by CaV2.1 (+44,47) and CaV2.1 (−44,47) Gln-40 were indistinguishable (Fig. 9C and Table 2). This contradicts with our previous report that polyQ expansion itself causes a hyperpolarizing shift of the inactivation curve for rabbit CaV2.1 channels carrying the β2 subunit (67). It is possible that the effect of polyQ expansion on VDI depends on species and β subtypes (68). When RIM2α was co-expressed, inactivation curves of CaV2.1 (+44,47) and CaV2.1 (−44,47) Gln-40 were indistinguishable (V_{0.5}, of CaV2.1 (+44,47) Gln-40 and CaV2.1 (−44,47) were -11.0 ± 2.2 mV and -6.7 ± 2.3 mV, respectively) (Fig. 9C and Table 2). These data indicate that polyQ expansion reduced the binding affinity of CaV2.1 C terminus for RIM2α but not the suppressive effect of RIM2α on the VDI of CaV2.1 (+44,47).

Functional impacts of α-RIMs on VDI of N-type CaV2.2 and R-type CaV2.3 channels

CaV2.2 VDCCs are a major source of presynaptic Ca2+ influx (69–71), and α-RIMs also interact with the C terminus of CaV2.2 (22, 57). To explore the generality of our findings using CaV2.1, associations of the C-terminal region of CaV2.2

J. Biol. Chem. (2017) 292(22) 9365–9381

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encoded by exons 41–47 of the CaV2.2 gene (CaV2.2 CTD) and the C-terminal region of CaV2.3 encoded by exons 41–48 of the CaV2.3 gene (CaV2.3 CTD) with RIMs were tested by co-IP experiments. Both the YFP-tagged CTD of CaV2.2 and the YFP-tagged CTD of CaV2.3 were co-immunoprecipitated with FLAG-tagged RIMs in HEK293T cells (Fig. 10A). Thus, the C-terminal interaction with RIMs is shared by CaV2.2 and CaV2.3.

We also examined the effect of RIMs on the VDI of N-type VDCCs containing the CaV2.2, CaV2.4, and CaV2.1 subunits and of R-type VDCCs containing the CaV2.3, CaV2.4, and CaV2.1 subunits. In CaV2.2-expressing cells, RIMs significantly shifted \( V_{0.5} \) values of the component susceptible to inactivation at high voltages (\( V_{0.5}^{\text{high}} \)) in inactivation curves of Ba\(^{2+} \) currents mediated by the deletion mutants of CaV2.2 (\(+44,47\)) co-transfected with vector, RIM1\(_{\alpha}\), and RIM2\(_{\alpha}\) were \(-46.2 \pm 3.0, -8.2 \pm 14.8, \) and \(-2.8 \pm 5.7 \) mV, respectively (Fig. 10B and Table 6), as reported previously (20). RIM2\(_{\alpha}\) induced significant reduction of low voltage-inactivated phases (the ratios of inactivating components of CaV2.2 co-transfected with vector, RIM1\(_{\alpha}\), and RIM2\(_{\alpha}\) were 0.64 \( \pm 0.11, 0.63 \pm 0.12, \) and 0.29 \( \pm 0.05 \), respectively) (Fig. 10B and Table 6). Suppressive effects of RIMs on VDI were also observed for CaV2.3 (the ratios of inactivating components of CaV2.3 with co-transfection of vector, RIM1\(_{\alpha}\), and RIM2\(_{\alpha}\) were 0.90 \( \pm 0.03, 0.63 \pm 0.09, \) and 0.67 \( \pm 0.08 \), respectively). We failed to detect signifi-
significant differences between the effect of RIM1 and RIM2 on VDI of CaV2.3 channels (Fig. 10B and Table 6). Thus, N-type CaV2.2 channels but not R-type CaV2.3 channels are susceptible to RIM2-mediated potentiation of VDI suppression.

Discussion

In presynaptic AZs, where SVs dock in close vicinity to VDCCs at the presynaptic membrane, depolarization-induced Ca$^{2+}$ influx via VDCCs triggers neurotransmitter release (1, 2). Previous proteomic analysis has shown that P/Q-, N-, and R-type VDCCs are embedded into protein networks assembled from a pool of 200 proteins (72). Unveiling the manner of protein-protein interactions in protein networks and their functional consequences is important for understanding the process of synapse formation and the modulation of synaptic transmission (73). Previously, we reported that α-RIMs increase neurotransmitter release by sustaining Ca$^{2+}$ influx through strong inhibition of VDI of VDCCs and by anchoring vesicles in the vicinity of VDCCs via the RIM-β subunit interaction (20, 39). It has also been reported that α-RIMs interact with the CaV2.1 C-terminal region and modulate VDCCs targeting to presynaptic AZs (22). These previous studies have shown that RIM-VDCC interactions are key to the protein assembly responsible for stimulus-secretion coupling in presynaptic AZs.

Multipoint interaction plays important roles in the regulation of properties such as stabilization of protein complexes. In RIM-VDCC complexes, the significance of multipoint interaction has not yet been resolved. To approach this question, the contributions of each interaction should be quantitatively assessed. Our data strongly indicate that RIM-α interaction is sufficient and necessary for α-RIMs to exert prominent suppressive effects of VDI of VDCCs, because in the CaV2.1 splice variant with the deletion of exons 44 and 47 (CaV2.1(−44,Δ47)), strong VDI suppression remains intact for RIM1α, but for RIM2α it is attenuated to a level comparable with that of RIM1α. In the presynapse, VDCCs display a weak VDI (13, 30). Moreover, in the calyx of Held nerve terminals of RIM1 and RIM2 conditional double knock-out mice, depolarization pre-pulses induced stronger inactivation of VDCCs compared with wild-type mice (48).

CaV2.1 is known to be inactivated through at least two voltage-dependent mechanisms (fast and slow inactivation) (74). The mechanism underlying inactivation is not completely understood but may involve “hinged lid” or pore block-type
α-RIMs diversify inactivation of CaV2.1 splice variants

![Diagram](image)

**Figure 9. Effects of RIM2α on VDI of polyQ-elongated P/Q-type CaV2.1 channels.** A, interaction of FLAG-tagged CaV2.1 CTD (+44–47) or (+44–47) Gln-40 (Q40) with YFP-tagged RIM2α in HEK293T cells. The interactions are evaluated by co-IP with monoclonal anti-FLAG antibody, followed by WB with polyclonal anti-YFP antibody. Input is 10% of the amount of cell lysate used for co-IP and is analyzed by WB using polyclonal anti-YFP antibody. B, quantification of the data shown in A. The intensity of the co-IP band for RIM2α normalized to the IP band for the CTD (CaV2.1 CTD (+44–47), 100%; CaV2.1 CTD (+44–47) Gln-40, 73 ± 18%; CaV2.1 CTD (+44–47) Gln-40, 73 ± 18%) (92). Data for nine experiments are presented in scatter plots. The **vertical error bars** represent standard deviations, and the mean values are indicated with **horizontal bars.***, p < 0.001; statistical significance of difference between CTD (+44–47) and CTD (+44–47) Gln-40. C, effects of RIM2α on inactivation curves of P/Q-type CaV2.1 (+44–47) Gln-40 currents in HEK293 cells expressing β2 and α2β1 subunits. The inactivation curves in cells co-expressing vector and CaV2.1 (+44–47) (dashed gray line) and in cells co-expressing RIM2α and CaV2.1 (+44–47) (dashed brown gray line) are taken from Fig. 3 and are shown for comparison. See Table 2 for statistical significance of the differences.

**Figure 10. Effects of α-RIMs on VDI of N-type CaV2.2 and R-type CaV2.3 channels.** A, interactions of YFP-tagged CaV2.2 CTD or CaV2.3 CTD with FLAG-tagged α-RIMs in HEK293T cells. The interactions are evaluated by co-IP with monoclonal anti-FLAG antibody, followed by WB with polyclonal anti-YFP antibody. Input is 10% of the amount of cell lysate used for co-IP and is analyzed by WB using polyclonal anti-YFP antibody. B, effects of α-RIMs on inactivation curves of N-type CaV2.2 and R-type CaV2.3 currents in HEK293 cells expressing β2 and α2β1 subunits. See Table 6 for statistical significance of the differences.

Mechanisms (75–77). It has also been reported that fast and slow inactivation represents structurally independent conformational changes (78). Our kinetic analyses of current decay showed that both RIM1α and RIM2α decreased the fast inactivation component and increased τslow regardless of the presence of the exon 44 and 47 regions in CaV2.1 (Fig. 5, C and D), although only RIM2α (but not RIM1α) increased τfast in the presence of the exon 44 and 47 regions in CaV2.1 (Fig. 5C). These findings may suggest that the interaction between CaV2.1 CTD and RIM2α induces conformational changes of the VDCC CaV2.1 α1 subunit in addition to those induced by the interaction between β subunit and α-RIMs.

By focusing on the splice variants of the CaV2.1 C-terminal region, we have deepened our understanding of the interaction between VDCCs and α-RIMs. α-RIMs interact with the CaV2.1 C-terminal region via the regions encoded by exons 44 and 47, the alternative splicing of which generates major C-terminal variants in the human cerebellum. Our experiments revealed at least four regions in the CaV2.1 CTD involved in interaction with RIM2α: a site encoded by exon 44 and three sites in the region encoded by exon 47. Analysis of relative mRNA levels of these CaV2.1 C-terminal splice variants indicates that 56, 86, or 67% of the total CaV2.1 mRNA carries either both exons 44 and 47, exon 44 alone, or exon 47 alone, respectively (Table 1). These spliced mRNAs are capable of encoding the CaV2.1 variants that interact with α-RIMs. The effect of RIM2α-CaV2.1 C-terminal interaction on VDI can be generated from 67% of the total CaV2.1 mRNA. Interestingly, there is a wide range of fundamental properties for individual synapses, including release probability, unitary response, and effects of previous stimulation on subsequent response (79). This suggests that CaV2.1 C-terminal splice variants may contribute to the heterogeneous molecular composition and function of VDCCs in...
As already mentioned above, among four regions in the CaV2.1 interaction between scaffolding proteins and that their competitive binding under-

CaV2 isoforms (9, 82–84). The scaffolding proteins and that their competitive binding under-
enriched in flexible regions of proteins that form conserved interaction surfaces to establish tissue-dependent protein-protein interaction networks (80). It is therefore possible that interactions among the CaV2.1 C-terminal region, the two regions of CaV2.1 CTD involved in interaction with RIM2α, the two regions of CaV2.1 CTD (the amino acid residues 2276–2292 and 2502–2505 of CaV2.1 (+44,47)) are highly conserved among the three CaV2 isoforms (supplemental Fig. 1). It has been shown that Bassoon localizes CaV2.1 but not CaV2.2 to AZs via molecular interaction with RIM-BPs (9), despite the fact that RIM-BPs can also interact with the PXPH motif of CaV2.2 (19). Furthermore, mutation of the PDZ-binding motif of EGFP-tagged CaV2.1 failed to affect the localization pattern of CaV2.1 in cultured mouse hippocampal neurons (86). These results indicate that the functions of some protein-protein interactions are dependent on other components and that use of appropriate assay systems and neuronal types is necessary to reveal their functionality.

From a pathological point of view, it is interesting that mutations associated with genetic diseases in the genes encoding RIMs modify their function in regulating VDCC currents (50, 51). In SCA6 patients, the relative mRNA level of the CaV2.2 splice variant, which possesses exon 47, is increased in cerebellar Purkinje cells but not in granule cells (87). In several episodic ataxia type 2 patients, mutations in CaV2.1 result in the loss of the regions encoded by exons 44 and 47 (32). These two diseases have similar symptoms, such as ataxic gait and loss of limb coordination. Although our results showed that the polyQ elongation itself does not affect functional regulation of VDCCs by RIM2α, increase in the relative proportion of the CaV2.1 splice variants that possess the region encoded by exon 47 may cause excessive RIM2α functional regulation. Dysregulation of the molecular organization of presynaptic AZs containing VDCC complexes may lead to abnormalities in different functional hierarchies of nervous system.

**Experimental procedures**

**cDNA cloning and construction of expression vectors**

Mouse RIM1α, mouse RIM2α, mouse CaV2.2, rabbit α6, human CaV2.1 (−44,Δ47), and rat CaV2.3 have been described previously (20, 39, 50, 88, 89). Human VDCC β4 subunit (GenBank™ accession number NM_001005747) was cloned using PCR from human whole-brain Marathon-Ready cDNA (Clontech) and was subcloned into pcDNA3.1(−) vector (Thermo Fisher Scientific). For production of YFP fusion proteins for RIM1α, RIM2α, CTD (amino acid residues 1855–2327) of CaV2,2 (GenBank™ accession number NM_001042528), and CTD (amino acid residues 1860–2295) of CaV2.3 (GenBank™ accession number NM_019294), cDNAs for these constructs and the YFP were subcloned together into the pCI-neo vector (Promega). For production of YFP fusion protein for β4, cDNAs for β4 construct and the YFP were subcloned together into the pcDNA3.1(−) vector. CTD (amino acid residues 1966–2505) of human CaV2.1 (−44,47)
α-RIMs diversify inactivation of Ca_{v}.2.1 splice variants

(GenBank™ accession number U79666) was cloned using PCR from human whole-brain Marathon-Ready cDNA (Clontech) and was subcloned into the FLAG-tagged vector pCMV-tag2 (Stratagene) and human Ca_{v}.2.1 (−44,Δ47) clone. To construct CTD of Ca_{v}.2.1 (+44,47) Gln-40, the Apal (7152)–MscI (7196) fragment containing 11 CAG repeats was replaced with synthetic oligonucleotides containing 40 CAG repeats. CTD of human Ca_{v}.2.1 splice variants with different combinations of exons 44 and 47 were constructed by PCR and were subcloned into pCMV-tag2 and human Ca_{v}.2.1 (+44,47) clone. Mutants or chimeras of CTD of Ca_{v}.2.1 and α-RIMs were constructed by PCR.

Cell culture and cDNA expression in HEK293 or HEK293T cells

HEK293 and HEK293T cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 30 units/ml penicillin, and 30 μg/ml streptomycin at 37 °C under 5% CO_2. Transfection of cDNA plasmidswas carried out using SuperFect Transfection Reagent (Qiagen). For electrophysiological measurements, recombinant plasmids were co-transfected with pRES2-EGFP (Clontech), and HEK293 cells with green fluorescence were analyzed. Transfected cells were grown for 36–48 h before electrophysiological measurements and co-IP assay.

Characterization of splice variation of Ca_{v}.2.1 C terminus in the human cerebellum by sequence analysis

We designed PCR oligonucleotide primers as follows: forward in the exon 42, 5′-GCTGGTCAACCTCACAAG-3′, and reverse in the exon 47, 5′-GCTGGGTTCCACTTACG-3′. Temperature cycles were as follows. Temperature initially 98 °C for 2 min was followed by 25 cycles at 98 °C for 10 s, and 68 °C for 1 min. The PCR products from human cerebellum cDNA (Takara, 9523) were electrophoresed on 1% agarose gel, cut out, purified using the Qiagen XL gel extraction kit (Qiagen), and ligated into the EcoRV-digested pBluescript II SK(−) (Stratagene). The ligation products were transformed into competent Escherichia coli DH5α cells and screened on Luria-Bertani plates containing ampicillin, X-Gal, and isopropyl β-D-1-thiogalactopyranoside for 15–20 h at 37 °C. White colonies were picked randomly and subjected to sequencing analyses using T7 and M13-reverse universal sequencing primers. The sequencing results were analyzed by using BioEdit software version 7.2.5.

Yeast two-hybrid screening and β-galactosidase assay

We subcloned CTD of human Ca_{v}.2.1 (+44,47) into pGBK-T7 and used it as a bait to screen a human brain pACT2 library (Clontech) in the yeast strain AH109 (Clontech). We plated transformants (2.5 × 10^6) on synthetic medium lacking adenine, histidine, leucine, and tryptophan and assayed His + colonies for β-galactosidase activity with a filter assay. Of the transformants, 56 were His +, and 6 of these were also LacZ −.

We isolated prey clone encoding amino acid residues 487–1349 of RIM2α (GenBank™ accession number NM_001100117).

co-IP assay in HEK293T cells

36–48 h after transfection, HEK293T cells were solubilized in Nonidet P-40 buffer (150 mM NaCl, 50 mM Tris, 1% Nonidet P-40, and 1 mM PMSF) and then centrifuged at 17,400 × g for 20 min. The cell lysate was incubated with anti-FLAG M2 monoclonal antibody (Sigma, F3165), and then the immunocomplexes were incubated with protein A-agarose beads (Santa Cruz Biotechnology, SC-2001), and the beads were washed with Nonidet P-40 buffer. The co-immunoprecipitated and immunoprecipitated proteins were characterized by Western blotting (WB) with anti-YFP antibody (Clontech, 632592) and anti-FLAG antibody (Sigma, F7425), respectively. Input was 10% of the amount of cell lysate used for co-IP and was characterized by WB with anti-YFP antibody. The chemiluminescence intensities of the bands were measured by Multigauge version 3.0 (Fuji film).

Current recordings

Whole-cell mode of the patch-clamp technique was carried out at 22–25 °C with an EPC-10 (HEKA Elektronik) patch-clamp amplifier as described previously (39, 41, 90). Patch pipettes were made from borosilicate glass capillaries (1.5-mm outer diameter, 0.87-mm inner diameter; Hilgenberg) using a model P-97 Flaming-Brown micropipette puller (Sutter Instrument Co.). The patch electrodes were fire-polished. Pipette resistance ranged from 2 to 4 megohms when filled with the pipette solutions described below. The series resistance was electronically compensated to >60%, and both the leakage and the remaining capacitance were subtracted by the −P/4 method. Currents were sampled at 10 kHz after low-pass filtering at 3.0 kHz (3 dB) in the experiments of inactivation kinetics and AP-like trains, otherwise sampled at 20 kHz after low-pass filtering at 3.0 kHz (3 dB). Data were collected and analyzed using Patchmaster (HEKA Elektronik) software. An external solution contained 5 mM BaCl_2, 148 mM tetraethylammonium chloride, 10 mM HEPES, and 10 mM glucose (pH 7.4-adjusted with tetraethylammonium-OH). The pipette solution contained 95 mM CsOH, 95 mM aspartate, 4 mM MgCl_2, 5 mM EGTA, 2 mM disodium ATP, 5 mM HEPES, and 8 mM creatine phosphate (pH 7.2-adjusted with CsOH).

Voltage dependence of inactivation

To determine the inactivation curve of VDCCs, Ba^{2+} currents were evoked by 40-ms test pulse to 10 mV after the 10-ms repolarization to −90 mV following 1-s (300 ms for Ca_{v}.2.3) prepulse voltage (V_{pre}) displacement (conditioning pulse) from −80 to 20 mV with 10-mV increments. Amplitudes of currents elicited by the test pulses were normalized to those elicited by the test pulse after a 1-s V_{pre} displacement to −80 mV. The mean values were plotted against potentials of the 1-s V_{pre} displacement. When the inactivation curve was monophasic, the mean values were fitted to the single Boltzmann equation, h(V_{pre}) = (1 − a) + a/(1 + exp((V_{0.5} − V_{pre})/k)), where a is the rate of inactivating component; V_{0.5} is the potential to give a half-value of inactivation; and k is the slope factor. Otherwise, the mean values were fitted to the sum of two Boltzmann equations: h(V_{pre}) = (1 − a − b) + a/(1 + exp((V_{0.5}^{\text{low}} − V_{pre})/k^{\text{low}})) + b/(1 + exp((V_{0.5}^{\text{high}} − V_{pre})/k^{\text{high}})), where a, b, and (1 − a − b) are the ratios of a low voltage-induced phase, a high voltage-induced phase, and a non-inactivating phase; V_{0.5}^{\text{low}} and V_{0.5}^{\text{high}} are the potentials that give a half-value of components.
susceptible to inactivation at low voltages in inactivation curves and at high voltages; and $k_{\text{low}}$ and $k_{\text{high}}$ are the slope factors. The decay phase of Ba$^{2+}$ currents evoked by 1-s test pulses was fitted by two (fast and slow) exponential functions with a non-inactivating component: $I(t) = a + b \exp(-\lambda_1 t) + c \exp(-\lambda_2 t)$, where $I(t)$ is the inactivating current as a function of time; $a$ is the current amplitude at $t = \infty$; $b$ and $c$ are the amplitudes of the time-dependent components; and $\lambda_1$ and $\lambda_2$ are the reciprocals of the fast ($\tau_{\text{fast}}$) and the slow ($\tau_{\text{slow}}$) time constants of inactivation, respectively (41, 91). APs began at $-80$ mV and peaked at $33$ mV. Rising and falling slopes were 283 and $-103$ V/s, respectively (20, 65). Leaks and capacitive transients were subtracted by a $-P/4$ protocol.

I-V relationships

The individual activation data were fitted to standard Boltzmann equation in the form $I_{\text{a}} = G_{\text{max}}(V_m - V_{\text{rev}})/(1 + \exp(-(V_m - V_{\text{0.5}})/k))$, where $G_{\text{max}}$ is the maximal conductance; $V_m$ is the membrane voltage; $V_{\text{rev}}$ is the $I_{\text{a}}$ reversal potential; $V_{\text{0.5}}$ is the half-activation potential, and $k$ is the slope factor.

Statistical analysis

All data are expressed as the means ± S.E. The statistical analyses were performed using Student’s $t$ test. A value of $p < 0.05$ was considered significant.

Author contributions—M. H. designed the study, conducted most of the experiments, analyzed the results, and wrote most of the paper. Y. T. collected preliminary data and constructed vectors. K. Y. and C. F. W. conducted and analyzed the experiments on co-IP. H. K. conducted the sequence analysis of PCR products from the human cerebellum cDNA library. T. K., M. X. M., T. P. S., M. R., M. D. W., and Y. M. directed the research, wrote the manuscript, analyzed, interpreted the data, and critically reviewed the manuscript.

Acknowledgments—We thank S. Kiyonaka, T. Numata, R. Sakaguchi, and N. Ogawa for experimental advice and helpful discussions.

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